Killer Yeasts as Antifungal Biocontrol Agents

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I certify that this thesis is a true and accurate account of my own research

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Abstract

The ability of killer yeast to inhibit the growth of fungi, specifically wood decay fungi, has been investigated. An initial broad screen of 17 yeast versus 17 fungi demonstrated that killer yeast can and do exhibit antagonistic as well as growth stimulatory affects on fungi. Yeast from the *Saccharomyces* and *Pichia* genera exhibited the greatest and broadest spectrum of antagonism towards fungi and two yeasts one from each genus were studied further, namely *Saccharomyces cerevisiae* K28 and *Pichia anomola* NCYC 750. Both yeast showed evidence of affecting fungal growth through the secretion of volatile chemicals. Partially concentrated cell free supernatant from the two yeasts also displayed antagonistic properties towards fungi demonstrating that a substance exhibiting antimycotic properties is secreted during the growth of the two yeasts. The mechanism by which this antimycotic activity was mediated by the yeast *Pichia anomola* NCYC 750 was identified to be associated with a 27 kDa exoglucanase enzyme. The possibility that the antimycotic activity observed is associated with yeast killer toxins is discussed.

Chapter 1

Introduction

1.1 Biological Control

1.11 Biological control beginnings

Biological control can be defined as the introduction of an organism into an environment or ecosystem in order to control or limit the amount of pest species present in that ecosystem. This definition has been revised in recent years to include the use of the product or products of an organism, as well as the organism itself, to control a pest species (Bruce 1995).

From a historical perspective the earliest recorded applications of biological control were in the period when European countries were expanding their colonies around the world and were suffering from large numbers of pest organisms in the non-native crops they were cultivating (Simmonds *et al.* 1976). Reports prior to European colonial times of attempted biological control using ants and the use of ladybirds to control aphids by the Chinese have been reported but very little data exists to support this.

These earliest applications of biological control were generally unscientific and not always entirely successful. One of these early trials involved the use of mongoose, which was introduced into the West Indies to control sugarcane pests (Simmonds *et al.* 1976). Unfortunately this application was not successful and the mongoose are now considered a pest species in their own right, eating chickens and decimating ground lizard populations. Other examples include the introduction of giant toad (*Bufo marinus)* into

Martinique in order to control pests in sugarcane and the introduction of lizards into Bermuda to control mosquitoes (Simmonds *et al.* 1976). Further information on these early crude biocontrol attempts can be found in accounts given by Sweetman (Sweetman 1958) and Hagen and Franz (Hagan K. S. and Franz J. M. 1973) and a summary of this early history is given by Simmonds (Simmonds *et al.* 1976).

More recently "science based" biological control has taken more of an ecological approach, with the use of the natural predators and parasites of pest organisms being used as the biocontrol organisms. It is generally accepted that the advent of this modern approach began at the end of the nineteenth century in the USA with the successful introduction of the Australian insect *Rodolia cardinalis* (now known as the vedalia beetle) in Los Angeles, California. The vedalia beetle was used to control the pest insect *Icerya purchasi* which was causing a disease in young citrus trees known as cottony cushion scale and threatening to decimate the citrus fruit industry in California (van den Bosch and Messenger 1973). Similar introductions of insects parasitic to pest insects have been undertaken throughout the USA and the rest of the world since this time with varying degrees of success and failure.

Some of the most noteworthy research in this area of biological control has been undertaken in Hawaii. This is due to the fact that almost all the crop plants grown in Hawaii are introduced species, as are their pests, which thus have no natural enemies. As such, more research and development has been undertaken and therefore success has been achieved with biological control in Hawaii more than anywhere else in the world (DeBach 1964). The many successes achieved through this pioneering Hawaiian work have greatly aided in the worldwide development of biological control.

From these early beginnings, biological control has progressed to use more than just insects to control pest or problem insects. The field of biological control now encompasses all areas of biological science and utilizes a great variety of organisms from vertebrates to viruses to control an equally diverse range of pest and problem causing organisms in a wide variety of situations from farming to construction.

1.12 **Biological control and microorganisms**

The idea of biological control using microorganisms has been around for many years and was first posed as a scientific hypothesis by Agostino Bassi in 1835 (cited in; Simmonds *et al.* 1976) who noted that silkworms suffered from a disease caused by a fungus. From this observation Bassi theorized that other fungal species could infect other insects and therefore could be used to control insect pests. The first recorded application of microorganisms as biocontrol agents was by Ellie Metchnikoff in Russia in 1879 (Cloyd 1999), although earlier anecdotal evidence exists of applications of fungi in the environment to control pest species (Steinhaus 1956). Metchnikoff's studies involved the use of the green muscardine fungus *Metarhizium anisopliae* grown on artificial media to control the larvae of the wheat grain beetle *Anisoplia austriaca.*

1.121 Fungi

The study of fungi as a biocontrol agent has evolved beyond these early experiments to the development of commercially viable products for the biocontrol of many insect species. Examples of these products include: *M. anisopliae* which is now a registered biocontrol agent of household cockroaches in the U.S. and the fungus *Beauveria bassiana* which trades under the names Mycotrol GH-OF and Mycotrol GH-

ES for the biocontrol of many pest insects in a wide variety of crops (Hoffmann and Frodsham 1993). Fungi have also been studied for the potential biocontrol of other fungi, such as plant pathogenic fungi and wood decay fungi (see below).

1.122 **Bacteria**

The development of bacteria as potential biological control agents also began with the control of insect pest species. Most of the early work on bacterial biocontrol focused on the use of a single bacterium *Bacillus thuringiensis.* This was mainly due to the observations of Dr Ernst Berliner (Berliner 1911) who studied the Mediterranean flourmoth *Anagasta kuehniella*, a now worldwide species, the larvae of which destroys flour and other grain products. Berliner identified the causative agent of a disease of this moth as the bacterium *B. thuringiensis*. The first practical experiments of Berliner's observations were performed in Hungary (Husz 1928) for the control of corn borer larvae and a commercial product (Sporeine) was available in France before 1938 (Jacobs 1951). The Second World War temporarily stopped developments until the 1950s when research in America on the potential of *B. thuringiensis* to inhibit the alfalfa caterpillar (Steinhaus 1951) refocused interest on the area. Subsequent research has shown that the bacterium acts through the production of toxic crystals, strain specific for different insects and harmless to plants and vertebrates (Fischer and Rosener 1959). The development of bacteria as biocontrol agents did continue during the war years with the successful introduction of the bacterium *Bacillus popilliae* in the Eastern USA to control the Japanese Beetle (Dutky 1963) Bacteria have also been studied in recent years as potential biocontrol agents of postharvest decay (see below) with bacteria from the *Pseudomonas* species proving to be the most effective biocontrol agents. *Pseudomonas syringae* is now

a commercial biocontrol product marketed as BIO-SAVE® by the Ecoscience Corporation for the post harvest biopreservation of Citrus fruit, potatoes and peas.

1.123 **Yeast**

Yeast although classified as fungi are, unlike the majority of fungal species, single celled organisms. Yeast are defined as a specific type of fungi generally Ascomycetes or Basidiomycetes that reproduce vegetatively by budding or through the formation of sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman 1996).

The use of yeast as biological control agents has been limited to a few studies in the potable alcohol industry involving the use of killer yeasts (see Section 1.232). Killer yeasts (discussed in Section 1.2) secrete a toxin which inhibits the growth of sensitive yeasts and therefore the use of killer yeasts in the alcohol production industry may help to prevent the contamination of fermentations by "foreign" yeasts and as such increase reliability of fermentations and reproducibility in the industry.

In recent years, the ability of yeast to prevent colonisation of decaying matter has been studied as a possible biocontrol application for the prevention of storage rots in fruit and vegetables (see below).

1.13 **Biological control of post harvest decay**

Losses in post harvest fruit and vegetable crops due to rot caused by fungi can be up to 50 % (Coursey and Booth 1972) of the total crop in underdeveloped countries and have been reported as high as 25% in the USA (US Department of Agriculture and Service 1965). These losses are much greater in exported fruits and vegetables than for domestic fruit and vegetable markets (due mainly to the long storage times required) and

this often limits or even prevents the export of those foodstuffs that are more susceptible to rot. These figures are probably underestimates as they are calculated for and by the exporters and distributors of fruits and vegetables and do not take into account any on-the-shelf or at-home losses. Therefore some method for the protection of fruits and vegetables from rot causing fungi would be of significant global economic importance.

The only forms of protection currently available to fruit and vegetable producers are either chemical based antifungal treatments or methods reliant on the long-term refrigeration and storage of exported goods. Refrigeration is limited in its usefulness due to the high associated cost, especially over long time periods such as those entailed during transportation by sea. Chemical options, although relatively cheap and effective, are also becoming unviable due to an increase in the occurrence of resistance to chemical fungicides in post harvest fungal pathogens (Delp 1980; Spotts and Cervantes 1986; Smith 1988) and the possible toxic hazards to, and an increasing awareness of, such hazards by consumers. As such a relatively inexpensive, non-hazardous method of fruit and vegetable preservation would be of great use to the global fruit and vegetable industry. There are two possible candidates for this method of preservation: either through the development and use of less/non toxic chemical preservatives (the rise in demand for organic produce may limit this) or in the development and use of post harvest treatments based on the principals of biological control.

Research into this field appears to have been limited until the 1980s with only three examples prior to this period given in reviews of the biocontrol of plant pathogens by Cook and Baker (Cook and Baker 1983) and Janisiewicz (Janisiewicz 1988). Two of these examples involved the protection of strawberries from *Botrytis* rot, the earliest

using three fungi isolated from strawberry plants as antagonists (Bhatt and Vaughan 1962; Bhatt and Vaughan 1963) and the later using *Trichoderma* species as the antagonist (Tronsmo and Dennis 1977). The third example dates back to the 1950s with the work of Gutter and Littauer (Gutter and Littauer 1953) who isolated *Bacillus subtilus* from citrus fruits and identified it as antagonistic to 13 fungal diseases of citrus fruit. The development of strains of pathogenic fungi resistant to pesticides appears to have stimulated research in the 1980s with many studies being undertaken into the biocontrol of postharvest decay fungi. This more recent research has taken up from the early work described above and has continued the development of fungi and bacteria as potential biocontrol agents (Wisniewski and Wilson 1992; Punja 1997) with at least one commercial product being developed using the bacterium *P. syringae* (see Section 1.122). The main focus of research however, has shifted to yeasts, due mainly to the following reasons:

I) Yeast and fungi are ubiquitous on plant surfaces especially on fruit surfaces,

II) The possibility that fungi isolated from plants may themselves be or become pathogenic has limited the scope of research into fungal/fungal antagonism,

III) Yeasts, unlike the majority of bacteria, can colonize fruit surfaces and survive for long periods in the relatively dry conditions involved in fruit transportation (Janisiewicz 1988),

IV) Yeasts can use available nutrients and colonize wound sites (potential infection sites) very rapidly (Janisiewicz 1988),

V) Pesticides appear to have a minimal impact on yeasts (Andrews 1981),

VI) The genetics of yeasts are relatively well understood and therefore genetic manipulations are possible,

VII) Yeasts and yeast products already have wide acceptance as additives to foodstuffs and are generally acceptable to the public.

A wide variety of yeast species have now been studied for their potential use as biocontrol agents. These yeasts have been tested against a number of different fungal pathogens on an assortment of fruit and vegetables. A summary of some of these yeasts, target organism and plant species being protected is contained in Table 1.1. Postharvest biological control with yeast has also been studied using a secondary supplemental treatment alongside the yeast in order to enhance the antifungal effects. Examples include:

- Thermotherapy – the use of heat treatment (water bath or dry heat) prior to inoculation of yeast (Jijakli *et al.* 1993a; Conway *et al.* 1999; Leverentz *et al.* 2000),

- The use of Ca^{2+} and Mg^{2+} supplements - calcium has been shown to inhibit postharvest decay in apples and potatoes (McGuire and Kelman 1986; Conway *et al.* 1988; Conway *et al.* 1991; Biggs *et al.* 1997)) to aid the inhibition of fruit decay fungi (McLaughlin *et al.* 1990; McLaughlin *et al.* 1992; Wisniewski *et al.* 1995; Droby *et al.* 1997; Conway *et al.* 1999).

- Harvested fruits are often coated with formulations of shellac, cellulose or Nu-Film-P (poly-1-P-Menthan) to protect the fruits from infection and desiccation.

Mixing a biocontrol agent with these coatings may improve biocontrol activity and lengthen efficacy times (Jijakli *et al.* 1993; McGuire and Hagenmair 1996; McGuire and Dimotroglou 1999).

- The use of low doses of an antifungal compound such as nisin, a polypeptide antibiotic (El-Neshawy and Wilson 1996), thiabendazole (TBZ) (Arras and Arru 1999) or iprodione (Chand-Goyal and Spotts 1996; Chand-Goyal and Spotts 1997; Qing-Fan and Shiping-Tian 2000) to supplement the yeasts biocontrol activity,

- The use of UV-C light to stimulate an immune response (phytoallexin synthesis) within the postharvest product (Stevens *et al.* 1997).

- The use of nutrient supplements (or analogues) such as 2-Deoxy-D-glucose has been studied in order to enhance the postharvest biocontrol activity of yeast (Janisiewicz *et al.* 1992; Jijakli *et al.* 1993a; El-Ghaouth *et al.* 2000).

- Genetic manipulation of potential biocontrol yeasts has also been undertaken although to date this has been used only for research purposes such as the easy identification of the biocontrol yeasts on fruits and not in order to increase any antagonistic behaviour (Chand-Goyal *et al.* 1999; Nigro *et al.* 1999).

The mechanism by which these antagonistic behaviours are mediated is not yet fully understood but the premise that biological control of post harvest decay is primarily the replacement of the natural (antagonistic) organisms which grow on fruit but have been removed during processing in order to prevent the growth of decay organisms

(Wilson 1989) is widely accepted. Many theories have been suggested for the mode of action by which biological control is mediated, these theories include:

i) Competition with the fungal pathogen for nutrients has been suggested in a number of studies (Droby *et al.* 1989; Chalutz and Wilson 1990; Chand-Goyal and Spotts 1996; Chand-Goyal and Spotts 1997; Filonow 1998). It has been shown that the ability of some yeasts to rapidly sequester certain nutrients can inhibit fungal spore germination (Blakeman and Brodie 1977). Research of postharvest biocontrol has found that *Debaryomyces hansenii* can only inhibit the growth of *Penicillium digitatum* in nutrient poor conditions (Droby *et al.* 1989) and that yeast utilization of ^{14}C labelled sucrose is superior to that of *Botrytis cinerea* conidia (Filonow *et al.* 1996). This hypothesis has been further supported by the findings that *B. cinerea,* examined after a yeast biocontrol experiment, showed cellular damage similar to that found in aged and nutrient deprived cells (El-Ghaouth *et al.* 1998).

ii) Exclusion of fungal growth through pre-emptive inoculation of yeast into wound sites has been suggested (Droby *et al.* 1989; Jijakli *et al.* 1993b; Mercier and Wilson 1994) due to the fact that in almost all published studies effective biocontrol could only be achieved with prior or simultaneous inoculation of yeast onto fruit.

iii) The stimulation of immune response in the plant/fruit. It is known that certain adverse treatments of plants can stimulate the production of "immune response chemicals", or phytoalexins. This process has been utilized through the use of cell

walls of the yeast *Saccharomyces cerevisiae* to stimulate a phytoalexin response in lettuce leaves and soybean cotyledons in order to protect the plants from infection by *Rhizoctonis solani* and *B. cinerea* (Reglinski *et al.* 1995). Induced resistance was suggested as part of the mode of action involved in the biological control of postharvest fruit decay by Wilson (Wilson 1989; Wilson *et al.* 1994) and has since been observed using the yeasts *Rhodotorula glutinis* and *Cryptococcus albidus* (live and dead cells) (Elad *et al.* 1994), *Candida fomata* (Arras 1996), *C. saitoana* (El-Ghaouth *et al.* 1998), and *Pichia guilliermondii* (Arras *et al.* 1998).

iv) Antibiotic production has been shown to be one of the modes of action by which the bacterium *Bacillus subtilis* inhibits growth in postharvest decay fungi. Antibiotic secretion has also been demonstrated in two yeasts or yeast like fungi (Yamasaki *et al.* 1951; Baigent and Ogawa 1960) and many yeasts are known to secrete toxins against other yeasts (see Section 1.2). However, cell-free culture filtrates of various yeasts known to inhibit postharvest disease fungi have been tested and shown no evidence for secreted antibiotic compounds (Droby *et al.* 1989; McLaughlin *et al.* 1992; Arras and Demontis 1996). (Antibiotic production may be a problem for potential biocontrol agents of postharvest decay as antibiotics in the food chain may have a deleterious effect on public health (Wilson and Wisniewski 1992)).

v) Direct attachment of yeast to fungi has been demonstrated to play an important role in interactions between fungi and other microorganisms (Douglas 1987).

Some yeast species, including yeasts from the *Saccharomyces* and *Candida* species, have been shown to prey on other yeasts and, in the case of the *Candida* species, the conidia of two mould fungi (*Aureobasidium pullulans* and *Penicillium chrysogenum*) through the production of haustoria (Lachance and Pang 1997). Such attachments of yeast to fungal hyphae and conidia have been observed in a number of postharvest biocontrol studies (Wisniewski *et al.* 1991; Arras and Demontis 1996; El-Ghaouth *et al.* 1998) and have been suggested as a screening method for potential biocontrol agents of fungi (Cook and Long 1995). It has also been demonstrated that the attachment of *C. famata* to the mycelia of *P. digitatum* result in the degradation of fungal cell walls mediated by the secretion of lytic enzymes and phagocytic activity (Arras 1996).

The actual mode of action of yeasts biocontrol activity against pathogenic fungi is probably a combination of a number of the above effects and almost certainly varies between yeast species and fungal pathogen.

This research has, in the last few years, lead to a number of commercial pilot studies including those performed by Droby (Droby *et al.* 1993), McGuire (McGuire 1994), Chand-Goyal (Chand-Goyal and Spotts 1997), Arras and Arru (Arras and Arru 1999) and Usall *et al.* (Usall *et al.* 2000). These pilot studies have resulted in the release of two commercial biocontrol product for post harvest decay, Yield Plus by Anchor Bio-Technologies of South Africa and Aspire licensed to Ecogen Inc a US based company.

Yeasts have also been studied as a potential biocontrol agents for the post harvest storage of grain (Paster *et al.* 1993; Petersson and Schnürer 1998). Postharvest grains, used as animal feeds, are often stored under relatively high moisture conditions as this is

less expensive than drying the grains (Petersson *et al.* 1999). These high moisture conditions are unfortunately good growth conditions for spoilage fungi. This problem can be combated by storage in airtight conditions to minimize oxygen levels but some spoilage fungi such as *Penicillium roqueforti* can survive in low oxygen environments. *P. roqueforti* is also a potential mycotoxin producer (Haggblom 1990; Ohmomo and Kitamato 1994) and therefore the prevention of growth of this organism is of significant importance. The potential of yeast as a biocontrol agent of spoilage fungi of grain was first demonstrated in the laboratory in the early 1990s (Bjornberg and Schnürer 1993) and effective *in vivo* fungal biocontrol using a variety of yeast was shown soon afterwards (Petersson and Schnürer 1995). Further research has concentrated on the yeast *Pichia anomala,* a natural inhabitant of wheat (Lacey and Magan 1991), and has demonstrated that this yeast can not only inhibit *P. roqueforti* in varying oxygen concentrations in pilot scale silo storage (Petersson *et al.* 1999) conditions but actually inhibits mycotoxin production (Petersson *et al.* 1998).

Table 1.1 Yeast studied as potential biocontrol agents of Postharvest Decay

Yeast Species	Target fungi / plant disease	Plant	Authors
Candida guilliermondii	*Penicillium digitatum*	Grapefruit	(McGuire 1994)
Candida guilliermondii	*Botrytis cinerea* Aspergillus niger Rhizopus stolonifer	Table Grape & Wine Grape	(Zahavi *et al.* 2000)
Candida maritime	*Lasiodiplodia theobromae*	Mango	(Michereff *et al.* 1997)
Candida oleophila	*Botrytis cinerea* *Penicillium expansum*	Apple	(El-Neshawy and Wilson 1996)
Candida oleophila	*Botrytis cinerea* *Rhizopus stolonifer*	Strawberry	(Lima *et al.* 1997)
Candida oleophila	*Penicillium digitatum*	Grapefruits	(McGuire and Hagenmair 1996)
Candida oleophila	*Penicillium digitatum* *Penicillium italicum* *Geotrichum candidum*	Oranges	(Droby *et al.* 1998)
Candida saitoana	*Botrytis cinerea*	Apple	(El-Ghaouth *et al.* 1998)
Candida sake *Pichia anomala*	*Botrytis cinerea* *Penicillium spp*	Apple	(Jijakli *et al.* 1993b)
Candida sake	*Penicillium expansum*	Apple	(Usall *et al.* 2000)
Cryptococcus humicola *Sporobolomyces roseus*	*Botrytis cinerea*	Apple	(Anderson *et al.* 1997)
Cryptococcus laurentii *Cryptococcus infirmo-miniatus*	*Penicillium digitatum* *Monolinia fructicola*	Sweet Cherry	(Chand-Goyal and Spotts 1996)
Cryptococcus laurentii *Cryptococcus infirmo-miniatus* *Rhodotorula glutinis*	*Penicillium expansum* & (Thiabendazole resistant) *Penicillium expansum*	Pears	(Suger and Spotts 1999)
Cryptococcus laurentii	*Fusarium sambucinum*	Potato	(Schisler *et al.* 1995)
Debaryomyces hansenii	*Monilinia fructicola* *Penicillium digitatum* *Rhizopus stolonifer*	Peach, Tangerine Tomato & Sweetpotato	(Stevens *et al.* 1997)
Debaryomyces hansenii	*Penicillium chrysogenum*	Table Grape	(Sharma *et al.* 1997)
Kloeckera apiculata	*Rhizopus stolonifer* *Botrytis cinerea*	Grape, Peach & Apple	(McLaughlin *et al.* 1992)
Metschikowia pulcherrima	*Monolinia laxa* *Botryotinia fuckeliana*	Peaches & Table grapes	(DeCurtis *et al.* 1996)
Pichia guilliermondii *Rhodotorula glutinis*	*Penicillium digitatum*	Citrus fruits	(Arras and Demontis 1996)
Pichia guilliermondii	*Aspergillus flavus*	Soya Bean	(Paster *et al.* 1993)
Pichia anomala *Pichia guilliermondii* *S. cerevisiae*	*Penicillium roqueforti* *Aspergillus candidus*	Wheat	(Petersson and Schnürer 1995)
Pichia anomala	*Botrytis cinerea*	Apples	(Grevesse *et al.* 1998a)
Pichia anomala	*Penicillium roqueforti*	Wheat, Rye, Oats	(Petersson and Schnürer 1998)
Pichia membranefaciens	*Rhizopus stolonifer*	Nectarine	(Qing-Fan and Shiping-Tian 2000)
Rhodosporidum toruloides	*Venturia inaequalis*	Apples	(Sharma and Bhardwaj 2000)

1.14 **Biological control of wood decay**

Wood is used for a variety of purposes ranging from building material for houses, furniture and fences to paper production. The biological nature of wood however leaves it vulnerable to degradation, discoloration and destruction by biological organisms such as fungi. In the past the methods used for protecting wood and therefore extending the lifetime of buildings, furniture and fences has been through the use of chemicals such as creosote and copper chrome arsenic which are toxic to the wood decay fungi. However, this practice is changing due to an increased public awareness of the potential environmental impact of these toxic chemicals, increasing legislation regulating the use of such chemicals and the disposal of wood treated with them (Talarek 1988)) and an increasing occurrence of resistance to these chemicals exhibited by the target fungi. Therefore, as in the case of post harvest decay of soft fruit (wood decay could be considered as post harvest decay as trees are an agricultural product and wood is the harvested product), a less toxic, publicly acceptable and effective method for wood preservation is needed. One method that could satisfy these criteria is the development and use of biological control agents.

Wood decay fungi are specialised fungi from the Basidiomycota and Ascomycota groups and can be split into three categories based on differences in their enzymatic activities. These categories are:

1) White rot fungi - These fungi usually attack hardwoods, progressively decreasing the strength of wood by breaking down the major components of the wood, cellulose, hemicelluloses and lignin leaving the wood a white colour. This break down of wood components can either be a simultaneous process or may involve

the preferential break down of one component over another. White rot fungi include *Armillaria mellea*, *Heterobasidion annosum*, *Trametes versicolor* and *Xylaria hypoxylon.*

2) Brown rot fungi – These fungi generally attack softwoods, only degrade the cellulose and hemicellulose components of the wood leaving the brown lignin intact and are all basidiomycete fungi including *Serpula lacrymans, Piptoporus betulinus* and *Postia placenta.*

3) Soft rot fungi – These fungi usually attack green (high moisture) wood and also only degrade cellulose and hemicellulose but can also alter the chemical composition and structure of lignin. They are generally the least adapted of the wood decay fungi only capable of growth in wet nitrogen rich conditions and include fungi from the *Chaetomium species.*

A fourth group of fungi is also of interest in terms of biological control and wood, these are the wood staining fungi such as fungi which do not degrade wood but discolour the wood sap, leaving the wood unusable for some purposes. Wood stains can be caused by a wide variety of fungi such as *Ophistoma picea, Sclerophoma pithyophila, Ophistoma* species and moulds from the *Trichoderma* and *Penicillium* species.

Biological control of wood decay is an especially difficult problem, as many factors must be taken into account. The first and foremost of these difficulties is that the control must, in most cases, be absolute, unlike post harvest and agricultural biocontrol, as any failure of a biocontrol agent used to protect building material may have outcomes far more serious than the financial difficulties experienced in agriculture, such as loss of life. This control must also be effective over much longer periods of time than for

agricultural and post harvest control as the lifetime of timber products is much longer than that of edible products. Secondly, and as a result of this first difficulty, the biocontrol organism must be capable of long term sustainable growth on the wood without causing damage to the structural lignocellulose matrix of the wood and whilst maintaining biocontrol activity. Wood is a relatively nutrient poor growth substrate especially low in available nitrogen and therefore any biocontrol agent must be capable of effective growth in low nutrient conditions and as timber is a non-living product any utilised nutrients will not be replenished. Supplementary nutrients could be supplied to any biocontrol agent but this adds additional difficulties as these nutrients would be available to any wood rotting organisms as well as the biocontrol organism, and may also require repeated applications as they are utilised. Thirdly, and again unlike the case of post harvest biocontrol, wood decay can simultaneously be caused by a number of different organism as discussed above and therefore the potential biocontrol agents must be capable of combating a wide range of organisms.

The earliest recorded research in this field was in the 1960s and was based on ecological observations made of competition between fungi in the forest environment (Leach 1939; Rishbeth 1963). This early work involved laboratory and field tests (Shields and Atwell 1963; Dick and Hutchinson 1966; Ricard *et al.* 1969; Toole 1971), is reviewed by Frietag *et. al.* (Freitag *et al.* 1991) and Schoeman *et. al.* (Schoeman *et al.* 1999). Subsequent studies focused on two fungal genera, namely *Trichoderma spp.* and *Scytalidium spp.*

Scytalidium spp. were examined due to their ability to produce a diffusible antibiotic agent (Strunz *et al.* 1972; Stillwell *et al.* 1973) and have been studied as

potential biocontrol agents since this time (Ricard and Bollen 1968; Klingstrom and Johansson 1973; Bruce and King 1983; Highley 1990).

Trichoderma spp. also produce a range of diffusible antibiotic compounds (Bruce *et al.* 1984; Claydon *et al.* 1987; Bruce and Highley 1991; Highley *et al.* 1997) but also exhibit mycoparasitic activity (Dennis and Webster 1971b; Elad *et al.* 1983; Elad *et al.* 1984; Elad *et al.* 1987; Gupta *et al.* 1999). Successful biocontrol activity has been achieved in laboratory studies using *Trichoderma* spp. (Highley and Ricard 1988; Dawson and Morrell 1991; Score and Palfreyman 1994; Highley *et al.* 1997; Brown and Bruce 1999; Brown *et al.* 1999) but few field trials have been undertaken (Bruce *et al.* 1984; Bruce and King 1986a; Bruce and King 1986b; Ejechi 1997). These field trials have generally been of limited success with none achieving a 100% success rate. Problems reported have ranged from incomplete colonisation of wood (Bruce *et al.* 1990) to a lack of long term efficacy and therefore a need for repeated treatment (Ejechi 1997). Bruce *et. al.* (Bruce and Highley 1991), however, found that *Trichoderma* isolated from wood seven years after treatment maintained the ability to inhibit wood decay fungi.

Some research has been undertaken into the potential of bacteria as a biocontrol agent of wood decay fungi (Seifert *et al.* 1987; Ejechi 1998) and wood staining fungi (Florence and Sharma 1990; Bezert *et al.* 1996; Kim and Morrell 1998; Silva and Morrell 1998). This research has had very limited success especially in terms of long term bioprotection probably due to poor colonisation of wood by the bacterium tested.

To date very little research has been undertaken into using yeast as potential biocontrol agents of wood decay fungi. One study, undertaken to determine the potential of killer yeast as biocontrol agents against a number of fungi including wood decay fungi

(Walker *et al.* 1995), identified some yeasts as inhibitors of fungal growth. A more recent study (undertaken with the aid of the author) determined that killer yeasts can inhibit fungal growth on agar plates and in a laboratory assay performed using wooden blocks (Payne *et. al.* in print; Payne *et al.* 2000).

1.2 Killer yeasts

1.21 The Killer Factor

The ability of yeast to prevent the growth of other yeasts was first noted in *S. cerevisiae* by Bevan and Makower (Bevan and Makower 1963) who divided yeast into three phenotypes, namely killer, sensitive and neutral. Killer yeast secrete a proteinaceous toxin lethal to sensitive strains but to which the killer yeast are immune. Since its discovery, this toxin based phenomenon, now known as the "killer" factor, has been studied intensively. These studies have shown that the killer factor occurs in various genera and species of yeast including *Cryptococcus* (Golubev and Shablin 1994), *Hansenula* (*Williopsis*) *mrakii* (Ashida *et al.* 1983), *Kluyveromyces* (Rosini and Cantini 1987), *Metschnikowia* (Farris *et al.* 1991), *Trichosporon* (Buzzini and Martini 2000a) *Williopsis* (Hodgson *et al.* 1995; Theisen *et al.* 2000). Other studies have shown that the range of killing activity varies between killer yeast (Rogers and Bevan 1978; Kazantseva and Zimina 1989; Buzzini and Martini 2000b) indicating that killer toxins are not identical and vary between yeasts. A survey performed by Young and Yagui (Young and Yagiu 1978) of cross reactivity amongst 20 killer yeasts from various genera identified that killer yeasts could be divided into 10 types (K^1 to K^{10}) based on killing activity

patterns and 10 types (R^1 to R^{10}) based on patterns of resistance to the ten killer types. When these two patterns were combined 13 classes of killer yeast were identified.

Killer yeasts have been reported in laboratory stocks and culture collections, (Philliskirk and Young 1975; Kazantseva and Zimina 1989) in collections of wine yeasts, (Naumova and Naumov 1973), in clinical and autopsy samples (Kandel and Stern 1979) and in the natural environment (Stumm *et al.* 1977; Buzzini and Martini 2000a). In recent years, killer yeasts have been found in such diverse environments as olive brines (Marquina *et al.* 1997), sediment samples from lakes (Vadkertiova and Slavikova 1995), vineyards and wineries (Farris *et al.* 1991; Neirotti *et al.* 1995) trees and cacti (Starmer and Phaff 1983) and salted cucumber (Yokoi *et al.* 1996).

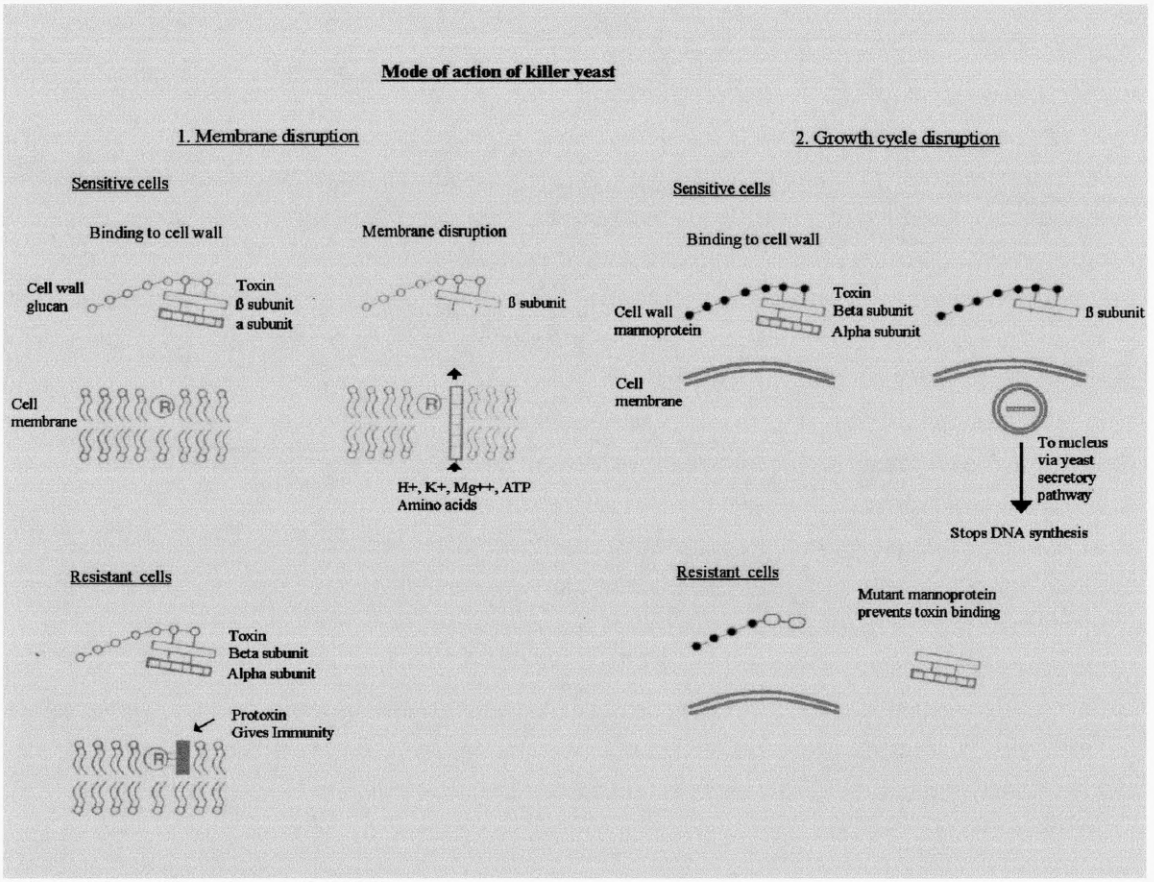
Due to this widespread distribution of the killer phenomenon, it is likely that it plays a role in the ecological development of microbial communities. Starmer (Starmer *et al.* 1987) suggested that the occurrence of killer yeasts in the natural environment was higher in decaying substances and especially in rotting fruit. Many yeasts are saprophytic organisms and are primary colonisers of decaying mater, as such any advantage these yeasts can gain over competitors would be selected for. Killer toxins are produced optimally during exponential growth when resources are abundant and require low pH for activity. Therefore, it can be surmised that the killer phenomenon is a potential mechanism for interference competition in which the production of the killer toxin prevents colonisation by competitors.

1.22 **Molecular biology of yeast killer toxins.**

All yeast killer toxins identified so far have been proteinaceous in nature. The molecular mechanisms involved in the expression of these proteinaceous killer proteins have yet to be identified in all yeasts. However, the systems in some yeast genera such as *Saccharomyces* (Woods and Bevan 1968; Mitchell *et al.* 1976; Skipper and Bussey 1977; DeLa Pena *et al.* 1981; Pfeiffer and Radler 1982; Schmitt and Tipper 1990; Goto *et al.* 1996), *Kluyveromyces* (Gunge 1986; Stark and Boyd 1986; Lehmann *et al.* 1987b; Vaughan Martini and Rosini 1989) and *Pichia* (Middelbeck *et al.* 1979; Worsham and Bolen 1990; Suzuki and Nikkuni 1994; Santos *et al.* 2000) have been studied. In all of the systems studied the killer toxins have been found to be proteinaceous in nature. The genetic material carrying the killer factor differs between genera and factor ranging from RNA (Mitchell *et al.* 1976; Pietras and Bruenn 1976; Sweeney *et al.* 1976; Fried and Fink 1978; Wickner 1979; Schmitt and Tipper 1990; Tipper and Schmitt 1991) and DNA plasmids (viruses) (Wesolowski *et al.* 1982; Gunge 1986; Worsham and Bolen 1990; Hayman and Bolen 1991) to chromosomal DNA (Chen *et al.* 1988; Goto *et al.* 1996) but the expression of the toxins appear to be similar. This genetic material, carrying the killer character, expresses a proteinaceous preprotoxin, which is larger than the killer toxin itself. This preprotoxin is proteolytically cleaved creating some form of active peptide toxin as well as an immunity determining peptide for the host cell (Douglas *et al.* 1988; Bussey *et al.* 1988a; Sturley and Bostian 1989; Bussey *et al.* 1990; Finkler *et al.* 1992; Suzuki and Nikkuni 1994). The toxin is then secreted via the yeast secretory pathway and exhibit modes of action reliant on either membrane disruption or inhibition of DNA synthesis (illustrated in Figure 1.1) (Bussey and Skipper 1975; Bussey 1981; DeLa Pena

et al. 1981; Hutchins and Bussey 1983; Douglas *et al.* 1988; Butler *et al.* 1991b; Schmitt *et al.* 1996). Some of the systems which have been studied so far are discussed below:

Figure 1.1 Mechanism of killer toxin activity



Adapted from (Douglas *et al.* 1988)

1.221 *Saccharomyces*:

The *Saccharomyces* genus is the most studied of the yeast genera and at least three types of killer system have been identified. These have included K1 (Bevan and Makower 1963), K2 (Woods *et al.* 1974) and K28 each of which is encoded by a linear dsRNA plasmid (virus). A K3 killer toxin was identified (Young and Yagiu 1978) but this has since proven to be a mutant of the K2 killer yeast (Wingfield *et al.* 1990).

The killer toxins of K1 and K2 *S. cerevisiae* exhibit many similarities relying on similar expression systems and sharing the same modes of action as they both bind to cell wall receptors, the 1,6-beta-D-glucan cell wall receptor (Hutchins and Bussey 1983) in the case of K1, of sensitive cells and disrupt cytoplasmic membrane functions causing the leakage of cytosolic components such as protons, ATP and amino acids, thus killing the cell (Skipper and Bussey 1977; DeLa Pena *et al.* 1981; Franken *et al.* 1998; Ahmed *et al.* 1999; Eminger *et al.* 1999; Vadaz *et al.* 2000). However, the two toxins do not exhibit the same killing profiles (Young and Yagiu 1978), have different genetic coding (Dignard *et al.* 1991) and do not share similar compositions of amino acids (Pfeiffer and Radler 1984). The K2 toxin has yet to be fully identified but is known to be glycoprotein of approx 16 kilodaltons (kDa) (Pfeiffer and Radler 1984) and the K1 toxin is also a glycoprotein and has been identified as a heterodimer with subunits of 9 kDa and 9.5 kDa (Bostian *et al.* 1984).

The K28 toxin is also a heterodimer protein and consists of 10.5 kDa and 10 kDa components (Schmitt and Tipper 1995). The toxin binds to a mannoprotein on the cell walls of sensitive yeasts (Schmitt and Radler 1987; Schmitt and Radler 1990) but rather than disrupting cell membranes the toxin inhibits DNA synthesis in order to kill sensitive

cells. Recent research has identified that after binding to cell walls the K28 killer toxin is taken into sensitive cells by endocytosis, the active toxin then travels a reverse path through the yeast secretory pathway and blocks DNA synthesis in the nucleus (Eisfeld *et al.* 2000) interrupting cells growth at the S phase of the cell cycle (Schmitt *et al.* 1996). Resistance appears to be mediated by mutations in the cell wall mannoprotein structure (Schmitt and Radler 1990).

1.222 *Kluyveromyces.*

In *Kluyveromyces* at least 6 species have been identified as possessing killer factor (Martini and Rosini 1989). The *K. lactis* killer toxin is the most studied of these toxins. This toxin is encoded on a two linear DNA plasmid (Wesolowski *et al.* 1982) and is a glycoprotein made up from three polypeptide subunits with molecular weights 99 kDa, 30 kDa and 27 kDa (Stark and Boyd 1986). The mode of action is not fully understood but the toxin is known to inhibit the cell cycle at the G1 phase (Butler *et al.* 1991c) This process is brought about through the action of the 99 kDa alpha subunit which has been shown to exhibit chitinase activity (Butler *et al.* 1991b), cells deficient in cell wall chitin have been shown to be immune to the toxin (Takita and Castilho 1993), and the 27 kDa gamma subunit arrests growth at the cell cycle G1 phase (Butler *et al.* 1991a). *K. marxianus (fragilis)* NCYC 587 produces a K6 killer toxin which is a non-glycosylated protein with a molecular weight of 42,313 (Izgu *et al.* 1999) of which the mode of action is unknown.

1.223 *Pichia*

Several killer yeasts have been identified in the *Pichia genus* from at least six species most of which appear to affect cell surface permeability. The killer toxin from *P. farinose* is a chromosomally encoded toxin which is a heterodimer made up of two subunits with molecular weights of 6300 and 7800 and affects calcium channels in cell membranes (Suzuki *et al.* 1997; Suzuki 1999; Suzuki and Shimma 1999). The toxin of *P. kluyveri* is also thought to be chromosomally encoded (Zorg *et al.* 1988) and affects cell membrane permeability (Kagan 1983) as does the toxin of *P. membranifaciens* CYC 1106 which binds specifically to the cell wall protein (1-6)-beta-D-glucan (Santos *et al.* 2000). The killer toxin of *P. acaciae* however exhibits similarities to the toxin of *K. lactis* in that it is encoded by linear DNA plasmids (Worsham and Bolen 1990), consists of three subunits (110 kDa, 39 kDa and 38 kDa), has an associated chitinase activity and arrests cells at the G1 phase of the cell cycle (McCracken *et al.* 1994).

1.224 *Williopsis*

Several killer yeasts have been identified in this genus including *W. saturnus var saturnus, W. mrakii.* In *W. mrakii* (previously known as *Hansenula mrakii*) the killer system is chromosomally inherited (Kimura *et al.* 1993) and the killer toxin affects cell wall beta-1,3-glucan synthesis of budding cells causing cytosolic leakage (Komiyama *et al.* 1996). The toxin of *W. saturnus var saturnus* is also a chromosomally encoded (Kimura *et al.* 1993) proteinaceous toxin that inhibits cell wall synthesis in the buds of sensitive yeasts facilitating loss of cytosolic components (Komiyama *et al.* 1998).

1.23 **Potential uses of the killer yeasts and their toxins**

Since the discovery of the killer factor in yeast various potential uses for the phenomenon have been suggested in the scientific literature, some of these are listed below and are summarised in Table 1.2:

Table 1.2 Potential uses of killer yeast

Field	Potential application
Biological research	Taxonomy/Biotyping
	Modelling biochemical pathways
	Genetic cloning vectors
Medicine	Biotyping
	Therapeutic use
Fermentation Industry	Protection of fermentations from contaminants
Agricultural Industry	Biological control of plant pathogens
	Biological control of postharvest decay
Timber Industry	Biological control of wood decay

1.231 Taxonomy and Biotyping.

Due to the nature and diversity of yeast killer systems and the complex killer, sensitive and neutral phenotypes found amongst yeast genera it has been suggested that the killer system could be used as a method for classifying and biotyping yeast species. This was first suggested by Rogers and Bevan (Rogers and Bevan 1978) who crudely grouped fourteen yeast strains into four classes depending on their sensitivity to different killer toxins. This idea was followed by Vustin *et al.* (Vustin *et al.* 1991) who used the killer toxin of *W. pratensis* to differentiate between basidiomycete yeasts on the basis of killer/sensitive relationships due to differing cell wall compositions and has been further developed since this time (Buzzini and Martini 2000b). The use of killer toxins for biotyping within species has also been suggested and studied in *Kluyveromyces* (Vaughan Martini *et al.* 1988) and *Candida* (Caprilli *et al.* 1985) and furthered with the use of other antifungal chemicals in conjunction with killer toxins (Lehmann *et al.* 1987a).

This taxonomic use of yeast killer toxins has not only been used to differentiate between yeasts but has also found applications for the biotyping of bacteria (Morace *et al.* 1989), actinomycetes (Morace *et al.* 1988) and mycelial fungi (Polonelli *et al.* 1987).

1.232 Fermentation industry.

The alcohol industry as a whole may be particularly susceptible to problems caused by killer yeasts through contamination of mashes and musts. If contamination occurs competition for nutrients and the possible killing of industrial yeast strains will cause the loss of production through off flavours and limited alcohol concentrations.

In the wine industry, contamination of musts by killer yeasts has been suggested as a cause of stuck fermentations (VanVuuren and Wingfield 1986) and it has been shown that concentrations of killer contaminants as low as 0.8% of the total yeast population can result in the total elimination of the fermentation yeast (Ramon-Portugal *et al.* 1998). However many wine yeasts have been found to exhibit the killer character (Naumova and Naumov 1973; Shimizu *et al.* 1985; Tredoux *et al.* 1986) and as such could be immune to killer contaminants and actively protect fermentations against yeast contaminants. As a result of this killer yeasts have been studied for their potential as a "biocontrol agent" for use in potable alcohol production (Yap *et al.* 2000). This has lead to the construction of wine yeasts through genetic manipulations (Boone *et al.* 1990; Gniewosz *et al.* 1999) and breeding programs (Hara *et al.* 1980) that carry one or more killer factors. These yeasts as well as limiting must contamination by killer yeasts also prevent contamination by other yeasts and are therefore of great industrial and financial importance for wine production.

The brewing industry has also suffered problems due to contamination by killer yeasts. Unfortunately though, killer character has not been observed in industrial brewing yeasts. Therefore, as with wine yeasts, genetic manipulations have been undertaken to introduce the killer system to brewing strains of yeast (Young 1981; Janderova *et al.* 1986). These "killer" brewing strains have been used in trial fermentations and have been found to successfully kill a range of contaminant yeasts (killer and non killer) and the beers produced are similar to those produced by control brewing strains (Young 1983; Hammond and Eckersly 1984).

Killer yeasts have also been isolated as contaminants of Sake fermentations (Imamura *et al.* 1974; Katohda *et al.* 1997) and the potential of killer yeasts to limit losses due to contaminants has led, as in the above cases, to the breeding (Nitta *et al.* 2000) (which incidentally possessed a high alcohol tolerance) and the genetic development (Yoshiuchi *et al.* 2000) of Sake yeast possessing the killer factor.

Problems due to toxicity of killer toxins to animals and humans have been found to be minimal (Pfeiffer *et al.* 1988; Polonelli *et al.* 1990) possibly due to the pH and temperature requirements for killer toxin activity being different from those encountered in the body. With the exception of the killer toxin of *S. cerevisiae* K28 killer toxins of *S. cerevisiae* are unstable above 25-30°C and pH 4.8 (Pietras and Bruenn 1976; Pfeiffer and Radler 1984; VanVuuren and Jacobs; Soares and Sato 2000). However, consumer thoughts on "killer" alcoholic beverages may still limit the use of these modified killer yeasts in the industry.

1.233 Biological and Medical.

Yeasts are eukaryotic organisms and as such share many similarities in cell cycle biochemistry with higher organisms. Therefore, yeast can provide a useful model for the study of many of the biochemical pathways of these higher organisms. In the case of killer yeasts, many similarities exist between the secretion and binding of yeast killer toxins and the production and activity of hormones and neuropeptides in animals (Chan *et al.* 1979; Sossin *et al.* 1989). The yeast killer toxin genes are carried by a plasmid in many killer systems and these plasmids have the potential to be used as cloning vectors for the study and production foreign proteins (Dignard *et al.* 1991).

Medically speaking killer yeasts may prove useful in the identification and biotyping of pathogenic yeasts as well as providing a useful therapeutic tool. The killer system has been found to be useful in the differentiation of *C. albicans* (Polonelli *et al.* 1983) into 512 strains depending on their sensitivity to nine killer yeasts. The differentiation of other pathogenic yeast strains has also been studied (Morace *et al.* 1984) and the possible use of the killer system for the identification and study of other pathogenic microorganisms such as fungi has been reported (Polonelli and Morace 1988; Walker *et al.* 1995). The use of yeast killer toxins as a therapeutic tool has been suggested by Polonelli *et al.* (Polonelli *et al.* 1986) who inoculated animals with the skin pathogens *Malassezia furfur and M. pachydermatis* and successfully treated the resulting infections with the killer toxin of *Hansenula anomala* (UCSC 25F). A *P. anomala* killer toxin has also been identified as a potential biocontrol tool for the treatment of diseases caused by *Pneumocystis carinii* (Seguy *et al.* 1998) although potential antigenic properties may limit the use of this toxin. The sensitivity of *C. albicans* strains to yeast killer toxins has also led to possible therapeutic applications using killer toxins from *W. mrakii* (Hodgson *et al.* 1995) and *W. californica* (Theisen *et al.* 2000). As well as the above the killer toxins from a variety of yeast have been found to be active against pathogenic Gram-positive bacteria presenting more potential therapeutic applications (Izgu and Tinbay 1997).

Chapter 2

Materials and methods

2.1 Organisms

2.11 Yeasts

The yeast species used in this research are listed in Table 2.1. Organisms were obtained from the University of Abertay Dundee culture collection. They were maintained on malt extract agar slopes, subcultured every three months and were stored at 4°C. Long term storage was undertaken by lyophilisation of a grown culture. The killer status of the yeasts is shown in Table 2.1 and was confirmed using the streak plate agar diffusion assay described in Section 2.4.

Malt extract broth (Oxoid) and the minimal liquid media EMM3 (see Section 2.24) were used for the batch culturing of yeast in varying volumes of 50 ml up to 20 litres.

2.12 Fungi

Wood decay fungi were obtained from the Scottish Institute of Wood Technology's (S.I.W.T.) culture collection and the personal culture collection of Dr. Nia White, University of Abertay Dundee. Two different groups of wood fungi were used, the decay fungi and the staining fungi. The decay fungi consist of the soft rot and wood rotting basidiomycetes whilst the staining fungi consist of the sap stainers and the moulds. Plant pathogenic fungi were obtained from the Scottish Crop Research Institute (S.C.R.I.). All the fungi used are listed in Table 2.2.

Wood decay fungi were maintained on 5%/2% malt extract agar (Section 2.21). The fungal cultures were grown at 20 to 25°C dependent on the individual fungi and were stored at 4°C. Subculturing was undertaken every three months.

Plant pathogenic fungi were maintained on pea agar (Section 2.22), were subcultured every three months and were stored at 4°C.

The abbreviations used in the Tables 2.1 and 2.2 below refer to the following culture collections.

NCYC = National Collection of Yeast Cultures (Norwich UK)

LKB = Laboratory of Kodama Brewery, Akita, Japan

DBVPG = Departemento de Biologie et Vegetale Perugia (Univ. of Perugia, Italy)

EMPA = Eidgenössische materialprufrüngs – und Forschungsanstatlt (Swiss Federal Laboratories for Materials Testing and Research)

IMI = International Mycological Institute

FPRL = Forest Products Research Laboratory, original home of the UK National Collection of Wood-Rotting Fungi

ATCC = American Type Culture Collection

Table 2.1. Yeast strains employed in this research

YEAST	STRAIN NUMBER	KILLER STATUS
Saccharomyces cerevisiae	S 381	Killer [4]
Saccharomyces cerevisiae	K 28	Killer [3]
Saccharomyces cerevisiae	NCYC 1001	Killer [1]
Saccharomyces cerevisiae	NCYC 1006	Sensitive [2]
Williopsis mrakii	LKB 169	Killer [2]
Williopsis mrakii	NCYC 500	Killer [1] [2]
Williopsis saturnus	NCYC 22	Killer [4]
Williopsis saturnus	NCYC 23	Killer [4]
Pichia anomala	NCYC 432	Killer [2]
Pichia anomala	NCYC 434	Killer [1]
Pichia anomala	NCYC 435	Killer [1]
Pichia anomala	NCYC 750	Killer [2]
Debaryomyces vanrijii	NCYC 577	Killer [1]
Kluyveromyces marxianus	NCYC 587	Killer [1] [2]
Debaryomyces hansenii	DBVPG 7036	Killer [4]
Debaryomyces hansenii	DBVPG 7037	Killer [4]
Candida glabrata	NCYC 388	Killer [1]

Killer status from - [1](Young and Yagiu 1978), [2] (Hodgson *et al.* 1995) [3] (Schmitt and Radler 1987) [4] personal communication

Table 2.2 Fungi employed in this research

Fungi	Source / Strain number	Wood decay type/Plant pathogen.
Chaetomium globosum Kunze : Fries	EMPA 195 (ATTC 6205)	Soft rot fungi
Humicola grisea Traaen	EMPA 327	Soft rot fungi
Phialophora mutabilis (van Beyma) Gams et McGinnis	SIWT	Soft rot fungi
Petriella setifera (Schmidt) Curzi	SIWT	Soft rot fungi
Cephaloascus fragrans Hanawa	IMI 068865	Sap staining fungi
Ophoistoma picea (Munch) Sydow et Sydow	IMI 086982	Sap staining fungi
Sclerophoma pithyophila (Corda) von Hoehnel	IMI 020171	Sap staining fungi
Trichoderma harzianum Rifai	IMI 206040	Wood staining mold
Trichoderma saturnisporum Hammill	IMI 14685	Wood staining mold
Trametes versicolor (Linnaeus : Fries) Pilat	FPRL 28G	White rot fungi
Lentinus lepideus (Fries : Fries) Redhead et Ginns	FPRL 7H	Brown rot fungi
Gloeophyllum trabeum (Persoon : Fries) Karsten	FPRL 108 N	Brown rot fungi
Postia placenta (Fries) Larson et Lombard)	FPRL 280	Brown rot fungi
Botryodiplodia theobromea Patouillard	SIWT	Fruit rot, Sap staining fungi
Botrytis cinerea Persoon	SCRI	Plant pathogen (Noble rot in grapes)
Phytophthora infestans (Montagne) de Bary	SCRI	Plant pathogen (Potato Blight)
Phlebia radiata Fries	Wood Isolate	White rot fungi

2.2 Media employed for yeast and fungal culture

2.21 5%/2% Malt extract agar (personal communication)

Malt extract, 50g (Oxoid) and 20g of agar (Oxoid technical no.3) were mixed in 1000 ml of deionised water and boiled to dissolve the agar. This was then autoclaved at 121°C 15psi for 15 mins and after cooling to 55° was dispensed in 15ml amounts into 90 mm Petri dishes.

Slopes of malt extract agar were made by dispensing 10ml amounts of the boiled agar into Universal bottles which were then autoclaved at 121°C 15psi for 15 mins. These tubes were then cooled at an angle to achieve a sloped agar surface.

2.22 Pea agar (personal communication)

Frozen peas (125g) were placed in 500ml of water and boiled for 5 mins. The peas were then macerated in a blender and strained through muslin in order to remove any remaining large solid matter. The resulting filtrate was then made up to 1000ml with deionised water, 15ml of agar (Oxoid technical no 3.) were added and the pH was adjusted to 6.25. The media was then autoclaved at 121°C, 15psi for 15 mins and after cooling to 55°C 15ml amounts were dispensed into Petri dishes.

2.23 Methylene blue agar (Hodgson *et al.* 1995)

This media was prepared in citrate phosphate buffer (see Section 2.31) at pH 4.5 as follows:

To 1000ml of buffer the following was added.

1. 20g of bacteriological agar (Oxoid),

2. 20g of Sabouraud Liquid media (Oxoid),

3. 10g of Tryptone (Oxoid)

This was then boiled to dissolve the agar and the following were added.

4. 0.03g of methylene blue (BDH),

5. 50ml of glycerol

The resulting mixture was autoclaved at 121°C, 15psi for 15 mins. After cooling 15ml amounts were dispensed into Petri dishes as described in the Streak-Plate Agar Diffusion Bioassay below.

2.24 **EMM 3 Minimal media** (Creanor and Toyne 1993)

A minimal medium was used in order to facilitate the purification of killer toxin from growth media. The media selected was Edinburgh Minimal Media 3 (EMM3) which was made up as follows.

A: To 748ml deionised water the following was added:

Ammonium sulphate	5.00g
Sodium hydrogen orthophosphate	1.42g
Magnesium sulphate	0.50g
Potassium hydrogen pthalate	3.06g
Calcium chloride	0.01g
Potassium chloride	1.00g
Sodium sulphate	0.30g

This was then autoclaved at 121°C, 15psi for 15 mins.

B: 20g glucose was dissolved in 250ml deionised water and autoclaved at 121°C, 15psi for 15 mins.

C: Micronutrient mix 1000X stock

To 1000ml deionised water the following was added:

Boric acid	0.50mg
Manganese sulphate monohydrate	0.40mg
Zinc sulphate heptahydrate	0.40g
Iron chloride hexahydrate	0.20mg
Molybdic (VI) acid	0.16mg
Potassium iodide	0.10mg
Copper sulphate pentahydrate	0.04mg
Citric acid	1.00mg

D: Vitamin mix 1000X stock.

To 1000ml deionised water the following was added:

Inositol	10.00mg
Nicotinic acid	10.00mg
Calcium pantothenate	1.00mg
Biotin	0.01mg

Salts and glucose media were mixed and 1ml each of 1000X stock micronutrients mix and vitamins mix were added to make 1L of media.

2.3 Buffers

2.31 Citrate phosphate buffer

Citrate phosphate buffer (McIlvaine 1921) was made up using two stock solutions which can be mixed in varying ratios to make buffers in a range of pHs as shown in Table 2.3.

Table 2.3 Citrate phosphate buffer – Quantities of solution A and solution B plus 50ml of H_2O needed to make buffers from pH 3 to pH 7

Solution A (ml)	Solution B (ml)	Final pH
39.8	10.2	3.0
30.7	19.3	4.0
27.25	22.75	4.5
24.3	25.7	5.0
17.9	32.1	6.0
6.5	43.6	7.0

Solution A - 0.1 M solution of citric acid (21.01g Citric acid (Oxoid) in 1000ml H_2O)

Solution B - 0.2 M solution of dibasic sodium phosphate (53.65g of $Na_2HPO_4.7H_2O$ (BDH) in 1000ml H_2O)

Buffers at pH 4.5 and pH 5 were used in this study

2.32 **Citrate buffer**

Citrate buffer (Lillie 1965) was made up using two stock solutions which are mixed in various ratios to make up buffers at pHs ranging from 3 to 6.2 as illustrated in Table 2.4.

Table 2.4 Citrate buffer – Quantities of solution A and solution B plus 50ml of H_2O needed to make buffers from pH 3 to pH 6

Solution A (ml)	Solution B (ml)	Final pH
46.5	3.5	3.0
33.0	17.0	4.0
26.75	23.25	4.5
20.5	29.5	5.0
9.5	41.5	6.0

Solution A: 0.1 M solution of citric acid (21.01g in 1000ml H_2O).

Solution B: 0.1 M solution of sodium citrate (29.41g $C_6H_5O_7Na_3.2\ H_2O$ in 100ml H_2O)

A buffer at pH 4.5 was made for use in this study.

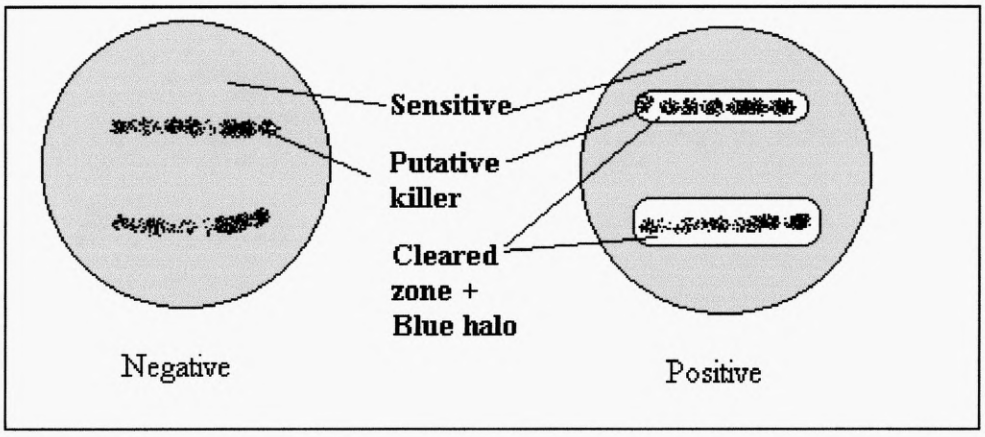
2.4 **Streak plate agar diffusion bioassay**

The streak plate agar diffusion bioassay as described by (Phillliskirk and Young 1975) was used in order to confirm and determine the killer status of the yeast species shown in Table 2.1. In order to determine the killer status of a particular yeast strain, a sensitive yeast was needed. Inhibition of the growth (together with evidence of fungicidal action) of this sensitive yeast would be indicative of the presence of a killer toxin(s) and therefore killer status could be assigned to a certain yeast. The sensitive yeast was *S. cerevisiae* NCYC 1006. Methylene blue dye was used in the assay in order to detect fungicidal activity in the form of dead cells. Methylene blue is taken up by yeast cells but only actively growing cells produce enzymes that can break down the dye, therefore dead cells retain the dye and stain blue. The assay was performed as follows:

The sensitive yeasts were grown in malt extract broth for 24 hours prior to the assay. Methylene blue agar was prepared as described previously (Section 2.23) and cooled to 45°C. One of the sensitive yeasts was then mixed with 15 ml of the molten methylene blue agar to give a seed inoculation of the agar of 5 x 10^4 cells/ml. The resulting mixture was immediately poured into a 90 mm Petri dish and swirled to ensure a thorough blending of the yeast and agar. After cooling the presumed killer yeasts were streaked (using a sterile cotton bud) in a single line onto the solidified agar (illustrated in Figure 2.1) and the plates were incubated at 25°C for 48 hours

The plates were examined, after incubation, for zones of clearing around the streaked "killers" surrounded by halos of blue stained dead cells, which would indicate killer toxin activity as illustrated in Figure 2.1.

Figure 2.1. Agar diffusion bioassay



2.5 **Agar diffusion bioassay to assess yeast antifungal activity**

A simple agar diffusion bioassay was developed for the initial screening of yeast versus fungi described in chapter 3 and is described below:

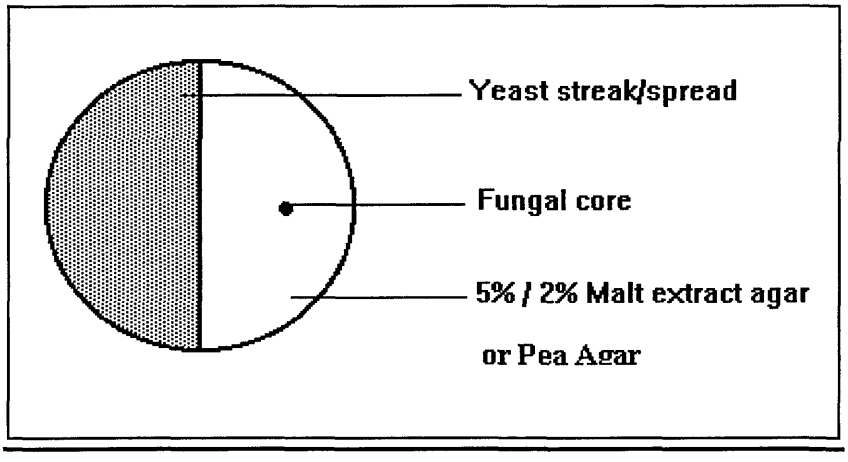
The fungi (Table 2.2) were grown on malt extract agar (for wood decay fungi) (Section 2.21) and pea agar (for plant pathogenic fungi) (Section 2,22) Petri plates for 5 to 10 days (depending on growth rates) prior to assaying.

Yeast were grown in 20 ml malt extract broth cultures for 24 hours. The yeast cultures were then spread plated onto an agar Petri plate (Malt extract agar, Oxoid) for a further 24 hours. A sterile cotton bud was then used to remove a quantity of yeast from an area of approximately 1cm by 4cm of the 24hr old plate. This yeast was then heavily smeared onto half of a fresh 90mm agar (Malt extract 5%/2%) plate. Two types of agar where used, malt extract agar (5%/2%) for assaying wood decay fungi and pea agar (supplemented with 1% glucose to facilitate yeast growth) for assaying plant pathogenic fungi. A 5mm fungal core was taken from the actively growing margin of the fungal cultures and placed onto the opposite half of the yeast smeared agar plate. An agar plate inoculated with a fungal core but containing no yeast was used as a control.

The plates were then incubated at 22°C for up to three weeks (dependent on the growth rate of the fungi being tested) until the fungi had entirely covered the control plate. Fungal growth was monitored at regular intervals (every3/4 days) by measuring the extension of the fungi towards the yeast and the overall area covered by the fungi. All of the agar diffusion bioassays were performed in duplicate.

The agar diffusion bioassay is illustrated in the diagram below (Figure 2.2):

Figure 2.2. Yeast *v.* fungi agar diffusion bioassay



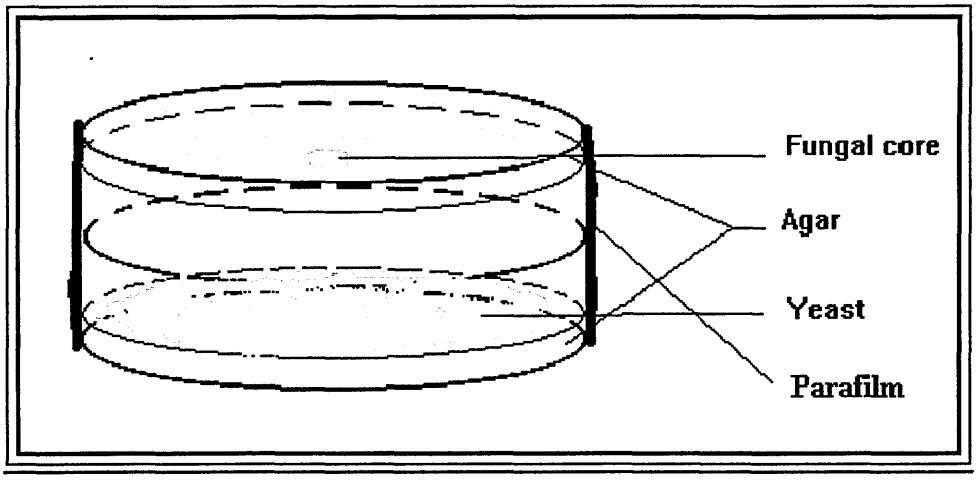
2.6 Assay of yeast volatile metabolites versus fungi

A simple assay similar to the agar diffusion assay described previously but allowing for diffusion of yeast volatiles through the air was used to examine the effect of yeast volatile chemical secretion on fungi as described in chapter 4. This assay was a modification of the fungal volatile assay originally described by Dennis and Webster (Dennis and Webster 1971a):

Fungi were grown on malt extract agar (5%/2%) for at least 5 days (depending on fungal growth rates) and yeast were grown in malt extract broth (Oxoid) for 24 hours prior to the assay ensuring that actively growing cultures were being tested. Each assay consisted of two 90mm malt extract agar plates. Yeast were inoculated onto one plate using a spread plating technique to form a "lawn", covering the entire plate, of actively growing yeast. The second plate was inoculated with a 4mm fungal core, taken from an actively growing fungal culture, in the centre of the agar. These two plates were sandwiched together with the yeast plate on the bottom and were sealed using Parafilm sealing tape, as illustrated in Figure 2.3. The yeast plate was placed on the underside of the "sandwich" in order to minimise the risk of contaminating the fungal plate by direct contact with the yeast.The plates were then incubated at 23° C for at 10 days or until the fungi reached the rim of the plates. Fungal growth was measured by radial growth from the core at regular intervals. All assays were performed in duplicate.

In order to assess the effect of media composition on volatile production the above experiment was also performed using minimal media for yeast growth. This minimal media was made up of EMM3 (Section 2.24) liquid media with the addition of 2% agar to solidify the media. The fungi were again placed on malt extract agar plates.

Figure 2.3. Volatile assay of yeast v fungi.



2.7 **Enzyme Assays**

2.71 **Exo-glucanase Assay**

A modification of the method described by Bruce (Bruce *et al.* 1995) was used to measure exo-glucanase (exo-ß-1,3-glucanase) activity, as follows:

Two test tubes labeled X and Y were used for each sample being tested. A 1ml sample of a 10 mg/ml solution of laminarin in 0.1M citrate buffer at pH 5 was added to each tube. One ml of the treated yeast supernatant samples (see Chapter 5, Sections 5.43 and 5.71) being tested was then added to tube X prior to both tubes being incubated at 40°C for 4 h.

After incubation 1ml of the sample being tested was added to tube Y. The glucose content in both tubes was then measured immediately using a Sigma glucose (HK) diagnostic kit. The difference in absorbance between tubes X and Y (X – Y) was then calculated. This figure was then used to express the exo-glucanase activity as µmols of glucose evolved per 1ml of sample per hour. All assays were performed in triplicate.

2.72 **Chitinase Assay**

The following method (Bruce *et al.* 1995) was used to determine chitinase activity:

Four tubes were used for each sample (treated yeast supernatant – see Chapter 5, Section 5.43), each containing 1ml of filtrate and 3.8mg purified chitin (Sigma - purified powder suitable for chitinase assay) in 1ml of citrate phosphate buffer pH 5 (Section 2.31). One tube from each set (A) was placed in a boiling water bath for ten minutes to denature any chitinase present prior to all four tubes being incubated for 24hr at 37°C.

After incubation all four tubes were placed in a boiling water bath for 10 min and 0.5ml was removed for assaying. Each 0.5 ml sample was placed in a labeled test tube (A – D) and 0.1 ml of 0.8M potassium tetraborate was added. The four tubes were then boiled in a water bath for 3 min before cooling. Three ml of p-dimethylaminobenzaldehyde reagent (DMAB) (10g DMAB in a mixture of 12.5 ml 10N HCl made up to 100ml with glacial acetic acid stored at 2°C and diluted 1:10 with glacial acetic acid before use) was then added to each tube and the tubes were incubated at 37°C for 20 min. After being left to cool each tube was mixed (vortexed) and an absorbance reading at 544nm was taken against a water blank which had been through the above assay procedure. The absorbance of tube A was then subtracted from the mean absorbance of the other three tubes (B – D).

A calibration curve was obtained by assaying a number of n-acetylglucosamine concentrations ranging between 0 and 125µg/0.5ml made up in citrate phosphate buffer (pH 5). Results were expressed as µmols of n-acetyglucosamine evolved per hour per ml of filtrate.

2.8 Polyacrylamide Gel Electrophoresis (PAGE)

Two PAGE gel systems were used for protein size identification in yeast supernatant samples obtained in Chapter 5: a conventional SDS-PAGE system and the NOVEX NuPage™ Bis Tris Electrophoresis system (Invitrogen, Paisley, UK).

2.81 **Conventional SDS-PAGE** (Section 5.5)

Gels with a 10% running gel and a 5% stacking gel were mixed as shown in Table 2.5. TEMED (the polymerizing agent) was mixed immediately prior to pouring the gels. The running gel was mixed, poured and left to set for 20 mins prior to the mixing and pouring of the stacking gel on top of the set running gel. Combs were inserted immediately after pouring the stacking gel and the gel was left to set for 20 mins.

Table 2.5 SDS-PAGE gel components

	10 % Running gel (ml)	5% Stacking gel (ml)
Acrylamide (29.26% acrylamide, 0.75% bisacrylamide)	26.7	6
Running gel buffer (18.18% Tris, O.4%SDS pH 8.9)	8	0
Stacking gel buffer (5.9% Tris, O.8%SDS pH 6.7)	0	9
dH_2O	45.3	21
$(NH_4)_2SO_4$	0.6	0.3
TEMED	0.04	0.015

Sample preparation.

All samples were prepared by mixing 10 µl of the concentrated putative mycocin (Section 5.5) with 10 µl of sample buffer (1ml stacking gel buffer, 0,8 ml 25% SDS, 0.5 ml ß-mercaptoethanol, 1 ml glycerol, 0.003g Bromophenol blue). This mixture was then boiled for 5 mins and centrifuged at 14000 rpm for 5 mins.

Gels were then placed in gel tanks (two gels per tank) containing enough tank buffer (31.6g TRIS, 20g Glycine, 5g SDS in 5000ml DH_2O) to ensure submersion of gels, were loaded with 20 µl of samples and were run at a current of 60 mA for 50 mins followed by 30 mA for 120 mins.

2.82 NOVEX NuPage™ Bis Tris Electrophoresis system (Section 5.8)

Precast NuPage gradient (4%/12%) gels were used.

Sample preparation: Protein samples were prepared using the protocols listed by NuPage – 62µl of each sample (pooled concentrated NCYC 750 fractions (1 to 3) and the unfractionated NCYC 750 toxin – see Section 5.8) was mixed with 25µl of 4X NuPage LDS sample buffer and 3µl of dH_20. 10µl of 10X Nu Page reducing buffer was added and the mixture was mixed immediately prior to incubation at 70°C for 10 minutes.

Pre cast gels were opened, washed and placed in the NuPage gel tank as per the NuPage instruction manual. Gels were then loaded with 20 µl of samples (prepared as above), the tank was filled with MES buffer and the gels were run at a constant 200 V and a start current of 120 mA per gel for 50 mins.

2.83 **Gel Staining**

Completed gels were stained using the Silver Staining method. This involved transferring of the completed gels between agitated baths containing the solutions listed in Table 2.6. The times shown in the right hand column are the minimum periods between transfers with the exception of those marked with an * which are precise exposure periods.

Table 2.6 Silver stain chemical and exposure times

Treatment	Chemical Components	Time
Fix	(Kodak Rapid Fixer)	60 (min)
Wash A	(50% ethanol)	20 (min) (X3)
Pretreat	(0.1g $Na_2S_2O_4.5H_2O$ in 500 ml dH_2O)	1 (min) *
Rinse X 3	(dH_2O)	20 (sec) * (X3)
Impregnate	(1g $AgNO_3$ + 0.375 ml 37% formaldehyde in 500 ml dH_2O)	20 (min) *
Rinse X 2	(dH_2O)	20 (sec) * (X2)
Develop	(30g Na_2CO_3 + 0.25ml 37% formaldehyde + 0.002g $Na_2S_2O_4.5H_2O$ in 500 ml dH_2O)	2 – 6 (min)
Rinse X 2	(dH_2O)	20 (sec) (X2)
Stop	(50% methanol, 12% acetic acid)	10 (min)
Wash B	(50% methanol)	20 (min)
Store	(50% methanol)	Up to 4 weeks

2.9 Soil moisture content measurement and adjustment

Soil moisture content was measured and adjusted for the soil studies described in Chapter 6 using the method described in the European standard DD ENV 807:1 (previously ENV 807:1993) and was performed as described below.

A 250 ml sample of soil was weighed (m_1) and placed in a beaker. The sample was then flooded with water and allowed to soak overnight. A coarse filter paper (Whatman no.1) was then placed over the base of a large Buchner funnel and the soaked soil sample was transferred onto this paper. Suction was then applied to the funnel until no more water could be seen to be withdrawn. The sample and filter paper were then placed in a preweighed beaker (m_2) and weighed (m_5). This beaker was then dried at 103 °C for 24 hours and reweighed (m_4). The mass of a wet filter paper after suction in a buchner funnel was then measured (m_6) and this filter paper was oven dried at 103 °C for 24 hours and reweighed (m_3).

The following calculations were then performed to calculate the water required to raise the moisture content of the soil to 95% of the soils water holding capacity.

1. Initial moisture content of soil (W_1)

$$\frac{(m_1+m_2+m_3)-m_4}{m_4-(m_2+m_3)} \times 100$$

2. Moisture content of soil at water holding capacity (WHC) (W_2)

$$\frac{(m_3+m_5)-(m_4+m_6)}{m_4-(m_2+m_3)} \times 100$$

3. Amount of water required to raise the soil moisture content to 95% of WHC

$$\frac{((W_3-W_2)/100)-W_1}{100+W_1} \times 100$$

W_3 = percentage moisture content required (95% in this case).

This process was performed in triplicate and the average result was used.

Chapter 3

Initial screen of yeast strains in order to detect those displaying antifungal activity

3.1 Introduction

In order to identify the extent and intensity of any antagonistic interactions between yeasts and wood decay fungi an initial screen was performed. This screen encompassed the seventeen yeast strains listed in Table 2.1 against the fourteen wood decay fungi and the three pathogenic fungi also listed in Table 2.2. The method used for this screening was a simple agar diffusion bioassay as described below. The results obtained were used to determine which yeast strains showed an ability to inhibit fungal growth and therefore which yeast/fungal interactions should be studied further.

3.2 Experimental Approach

The simple agar diffusion bioassay described in Section 2.5 was used in this initial screening of yeast versus fungi.

Measurements of the pH of the agar in the bioassays were also taken at regular intervals. The measurements were taken using a BDH pH measuring paper (sensitive to a range of pH 2 to pH 6 and calibrated using standard buffers) in the area between the fungal core and yeast smear (see Section 2.5 and Figure 2.2).

3.2 **Results of agar diffusion bioassays**

The results of the agar diffusion bioassay screens are displayed in Table 3.1 and are illustrated photographically in Figures 3.1 to 3.10.

Results showed large differences in interaction and intensity of interaction between yeast and fungi assayed. No single yeast was antagonistic to all of the fungi and no single fungus was affected by all of the yeast strains. Therefore, the interactions observed and reported below were specific to certain yeast and fungal strains.

Some yeast strains appeared to stimulate fungal growth as in the cases of *W. mrakii* NCYC 23 versus *Petriella setifera* and *D. hansenii* 7036 versus *Trichoderma harzianium.* In the case of *W. mrakii* NCYC 23 versus *P. setifera* fungal growth across the plate was greater than that of the control. This can be seen in the photograph of this bioassay (Figure 3.1) in which surface growth appears to be inhibited within 1 cm of the yeast but then reappears on top of the yeast. Other yeast appeared to have little or no effect on fungal growth as shown in the bioassays of *S. cerevisiae* NCYC 1001 versus *T. versicolor* and *C. glabrata* NCYC 388 versus *O. picea.* This effect is depicted in the photograph in Figure 3.2 which shows the bioassay of *S. cerevisiae* NCYC 1001 versus *T. versicolor.* In this bioassay fungal growth is slightly reduced across the plate but the presence of yeast appears to have little effect on the fungi which can be seen to be growing over the yeast.

Certain yeasts, however, appeared to be antagonistic to fungi. This antagonism varied greatly ranging from small to almost complete inhibition of fungal growth. In the case of the lesser antagonistic behaviour the fungi tested grew to within a few millimetres of the boundary of the yeast smear but no further. This response was seen in increasing

magnitude in the bioassays of *C. glabrata* NCYC 388 versus *T. versicolor, K. marxianus* NCYC 587 versus *C. globosum* and *S. cerevisiae* NCYC 1006 versus *P. mutabilis.* In these assays, illustrated photographically in Figures 3.3, 3.4 and 3.5, fungal growth clearly stops short of the edge of the yeast smear and appears to be less vigorous towards the yeast than away from the yeast. In the bioassay of *S. cerevisiae* NCYC 1006 versus *P. mutabilis* (Figure 3.5) this effect is more pronounced than in the previous two figures and shows positive inhibition of fungal growth.

This positive fungal growth inhibition was much greater in other bioassays with fungal growth being very limited in comparison with the control plate. These assays are illustrated in Figures 3.6 to 3.10 showing the following yeast/fungal interactions.

In Figure 3.6, showing the agar diffusion bioassay of *P. anomala* NCYC 750 versus *T. versicolor,* the antagonistic effect of the yeast on the fungal growth is clearly evident. The fungus appears to be inhibited in its growth, showing significantly less overall growth than the control and exhibiting greater growth away from the yeast towards the edge of the Petri plate than across the plate towards the yeast. A similar effect is evident in Figure 3.7 with *S. cerevisiae* K28 inhibiting the growth of *T. versicolor.* In this case growth appears to be inhibited in all directions with limited growth being visible in all directions.

Figure 3.8 shows the almost complete cessation of fungal growth caused by another *S. cerevisiae* yeast (strain S 381) on the soft rot fungus *C. globosum.* This figure also shows increased growth on top of the fungal core indicating vertical growth away from the agar. *S. cerevisiae* K28 also effected the growth of this fungi as is illustrated in

Figure 3.9 in which the direction of growth of *C. globosum* is almost entirely away from the yeast smear with very little growth being evident towards the yeast.

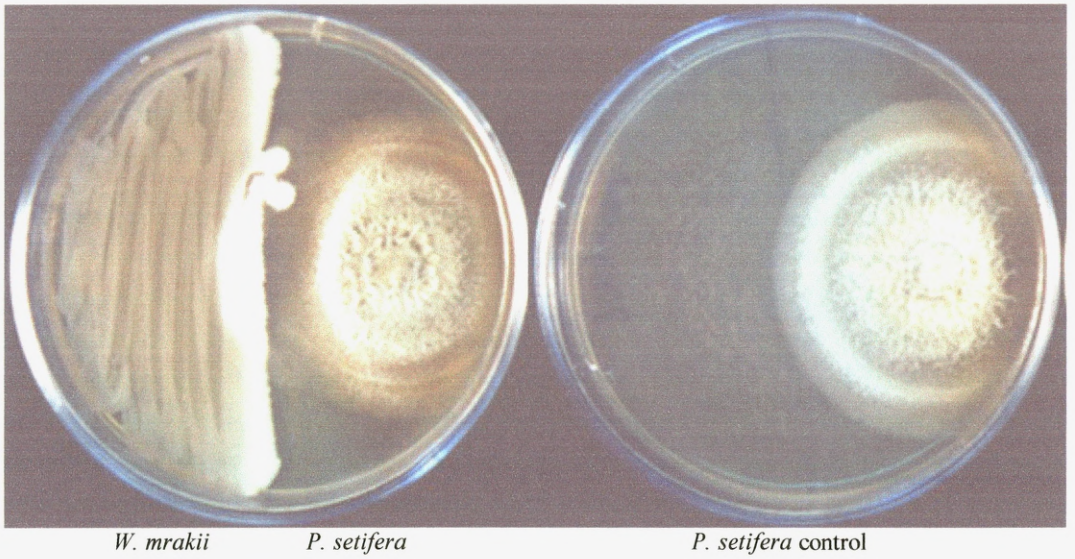
The *S. cerevisiae* strains assayed were not the only yeast strains to show extreme antagonistic affects on fungal growth, as was illustrated previously in Figure 3.7 by *P. anomala* NCYC 750. Other *P. anomala* strains also inhibited fungal growth and one of these strains, *P. anomala* NCYC 434 is shown in Figure 3.10. This figure again shows the majority of fungal growth to be away from the yeast smear and also shows what appears to be stimulated growth on top of and immediately around the fungal core.

The pH measurements recorded during these assays showed little change in the pH of the agar between the yeast and fungi with the pH across each Petri plate being recorded at approximately pH 4.1 (+/- 0.2) with a maximum pH drop across each plate (fungi to yeast) of 0.5.

Table 3.1 Yeasts v Fungi Agar Diffusion Bioassay.

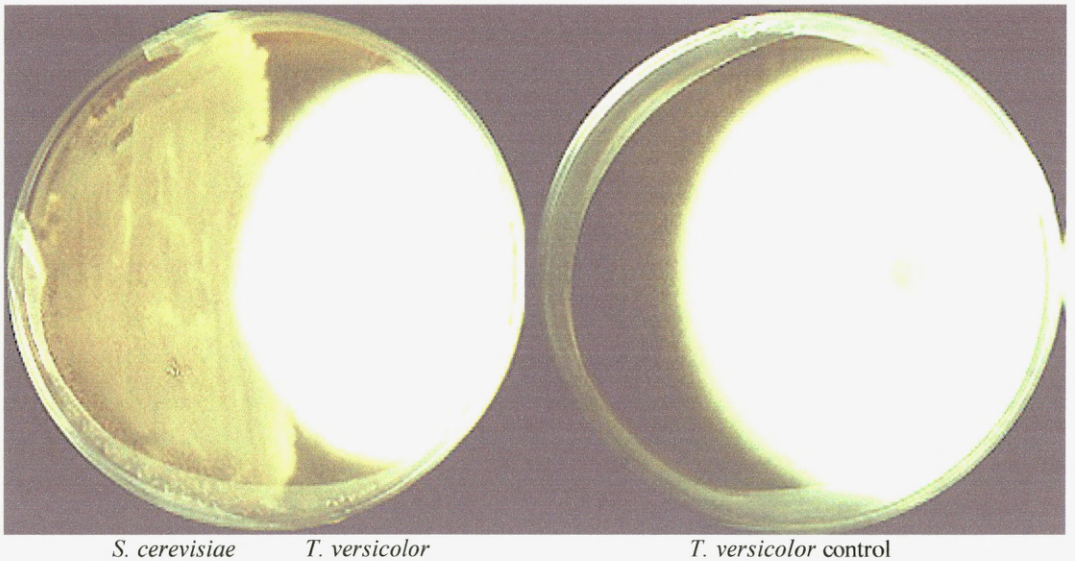
Killer yeasts	**Fungi**[1]																
	P. infestans	*B. cinerea*	*B. theobromae*	*S. pithyophila*	*C. cragrans*	*O. picea*	*T. saturnispourum*	*T. harzianium*	*H. grisea*	*P. mutabilis*	*P. setifera*	*C. globosum*	*G. trabeum*	*T. versicolor*	*P. placenta*	*L. lepideus*	*P. radiata*
S. cerevisiae 381	3	3	4	4	3	3	4	4	3	4	3	4	3	3	4	3	3
S. cerevisiae K28	2	3	3	2	4	3	4	3	3	3	3	4	3	4	4	3	3
S. cerevisiae NCYC 1001	3	3	2	4	3	4	4	4	3	3	4	3	4	2	4	2	3
S. cerevisiae NCYC 1006	3	5	2	4	4	3	4	4	4	3	4	4	3	3	3	2	3
W. mrakii LKB 169	3	1	2	2	3	2	2	1	3	2	2	1	2	2	2	2	2
W. mrakii NCYC 500	3	2	2	3	2	2	2	1	3	2	1	3	2	2	3	2	2
W. saturnus NCYC 22	2	4	2	3	2	2	2	1	3	2	1	3	2	2	3	3	2
W. saturnus NCYC 23	2	3	3	3	2	3	3	1	3	2	1	3	2	2	2	3	2
P. anomala NCYC 432	4	3	2	3	2	2	3	3	3	2	1	2	2	2	3	3	2
P. anomala NCYC 434	4	2	2	4	2	2	3	2	3	4	2	3	2	2	3	2	2
P. anomala NCYC 435	4	3	2	3	2	2	3	1	3	4	2	3	2	2	2	2	3
P. anomala NCYC 750	4	2	3	4	2	2	4	2	3	3	2	4	3	4	4	2	4
D. vanrijii NCYC 577	3	3	3	3	2	3	3	1	3	3	1	3	2	2	2	2	2
D. hansenii 7036	1	2	2	2	4	2	3	1	2	2	1	3	3	2	3	2	3
D. hansenii 7037	2	2	2	2	4	3	3	1	2	1	2	2	2	2	2	2	2
K. marxianus NCYC 587	4	3	2	2	2	1	2	2	3	2	1	3	2	2	2	2	2
C. glabrata NCYC 388	3	2	2	2	2	2	2	1	2	2	1	2	2	2	2	2	2

[1] Fungal growth inhibition (ranked from 1 to 5 relative to control)
1 = Growth stimulation 2 = No inhibition 3 = Some inhibition
4 = Good inhibition 5 = Little growth



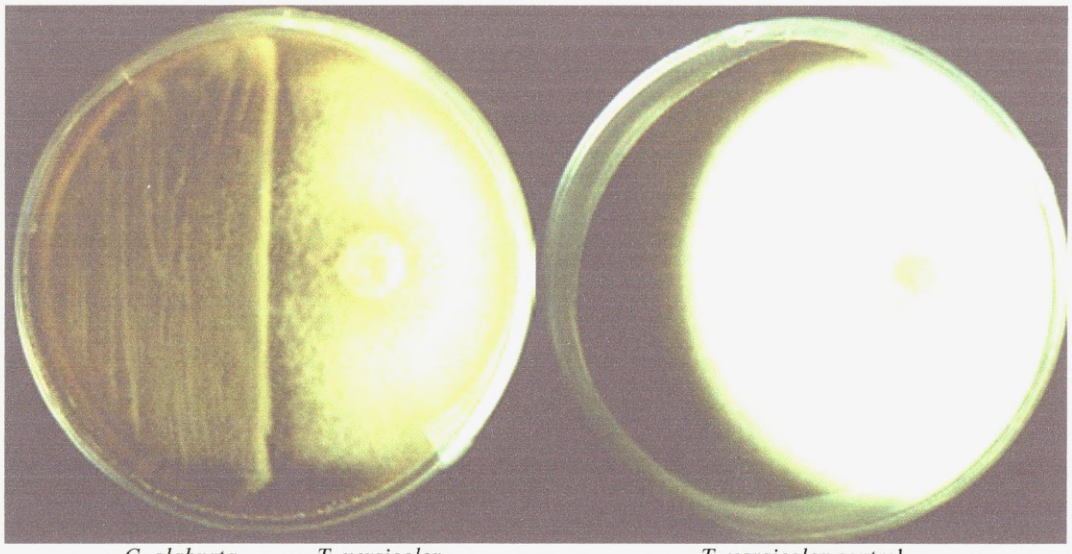
W. mrakii *P. setifera* *P. setifera* control

Figure 3.1 Agar plate diffusion bioassay of *W. mrakii* NCYC 23 versus *P. setifera*



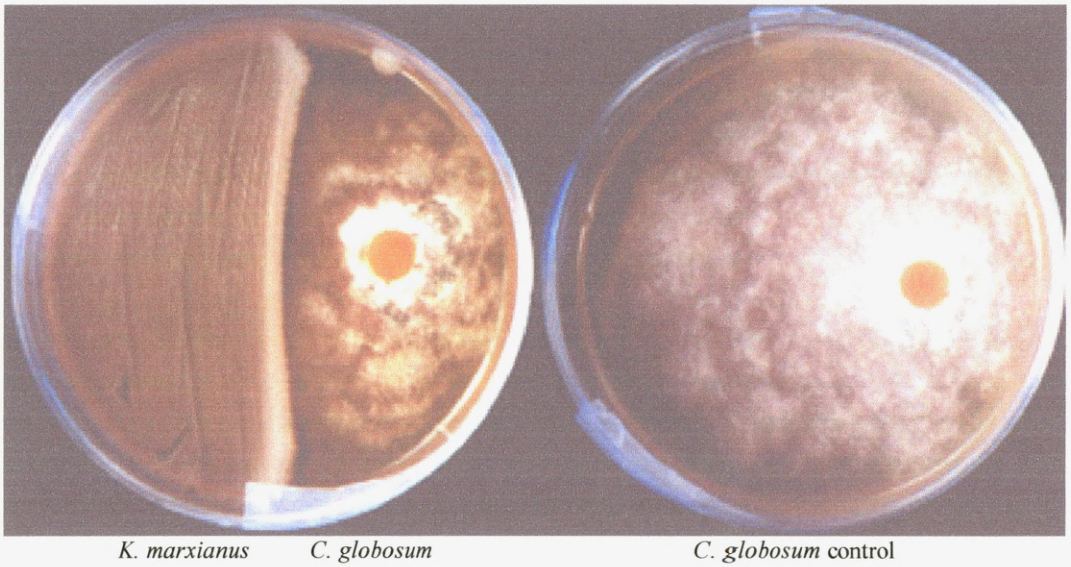
S. cerevisiae *T. versicolor* *T. versicolor* control

Figure 3.2 Agar plate diffusion bioassay of *S. cerevisiae* NCYC 1001 versus *T. versicolor*



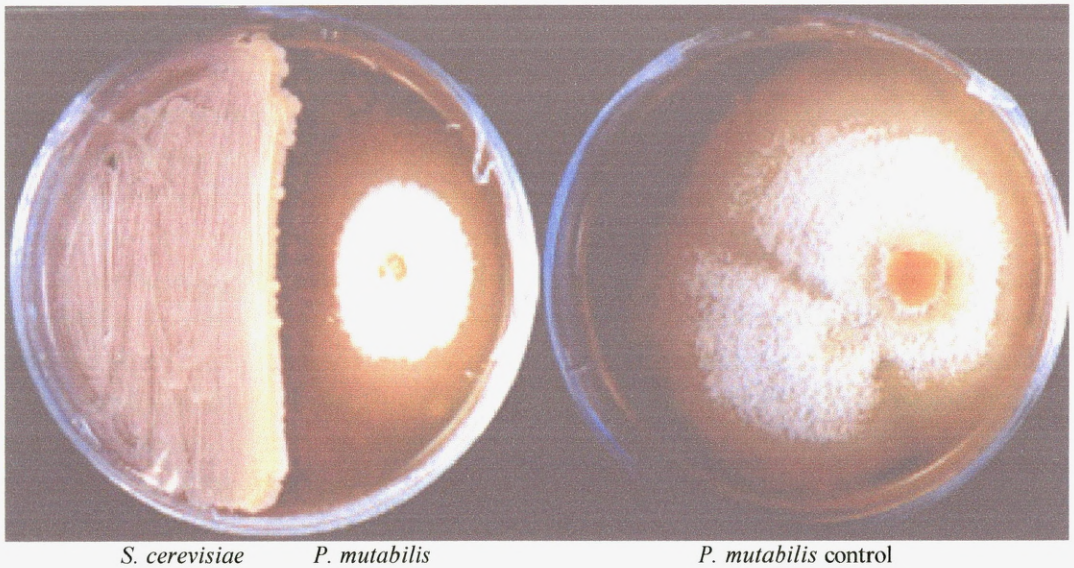
C. glabrata *T. versicolor* *T. versicolor* control

Figure 3.3 Agar plate diffusion bioassay of *C. glabrata* NCYC 388 versus *T. versicolor*



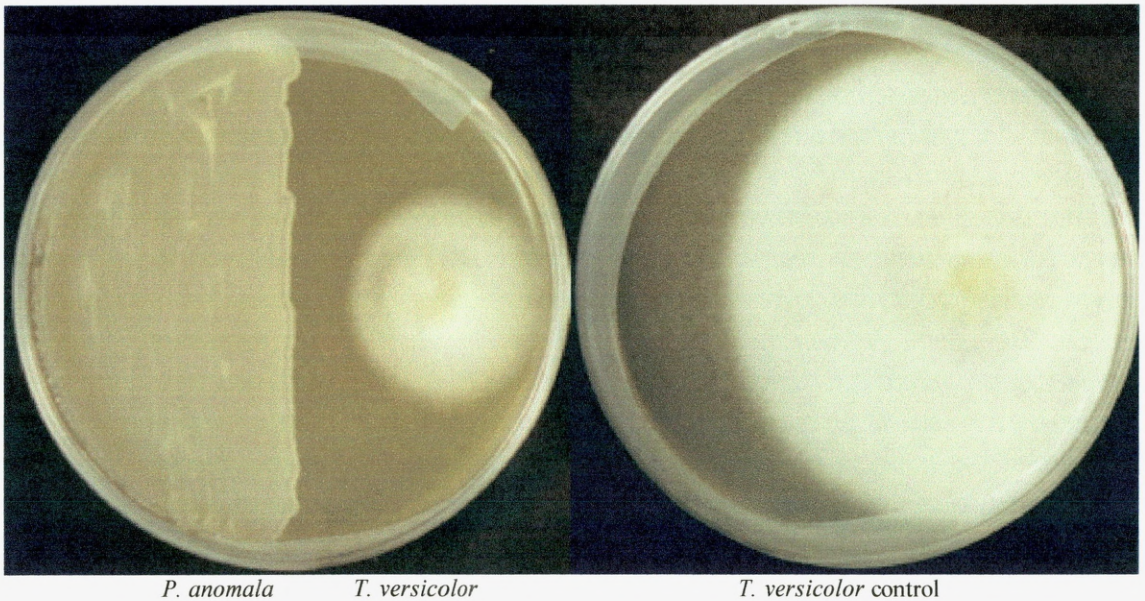
K. marxianus *C. globosum* *C. globosum* control

Figure 3.4 Agar plate diffusion bioassay of *K. marxianus* NCYC 587 versus *C. globosum*



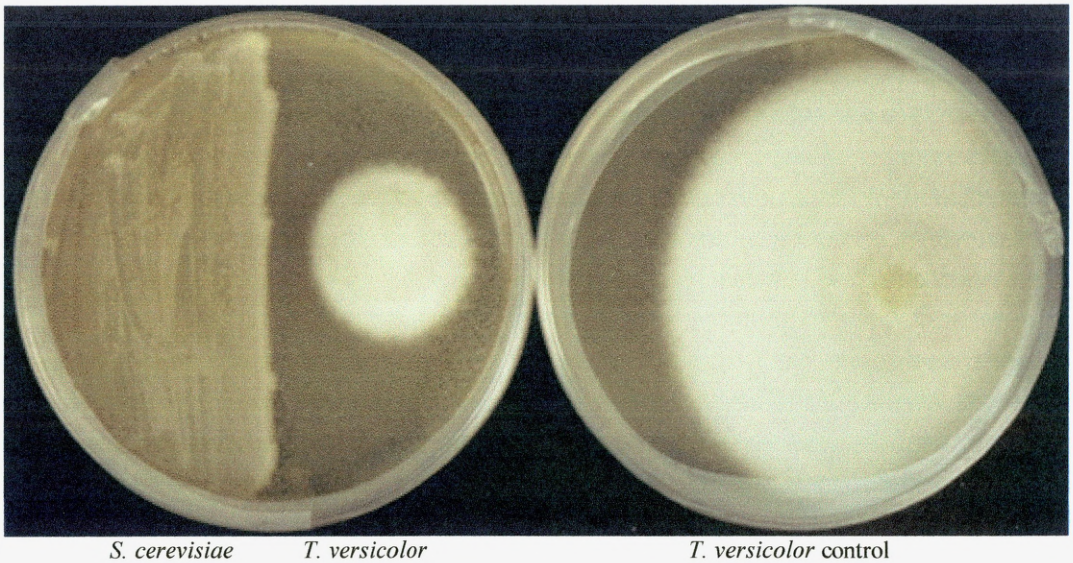
S. cerevisiae *P. mutabilis* *P. mutabilis* control

Figure 3.5 Agar plate diffusion bioassay of *S. cerevisiea* NCYC 1006 versus *P. mutabilis*



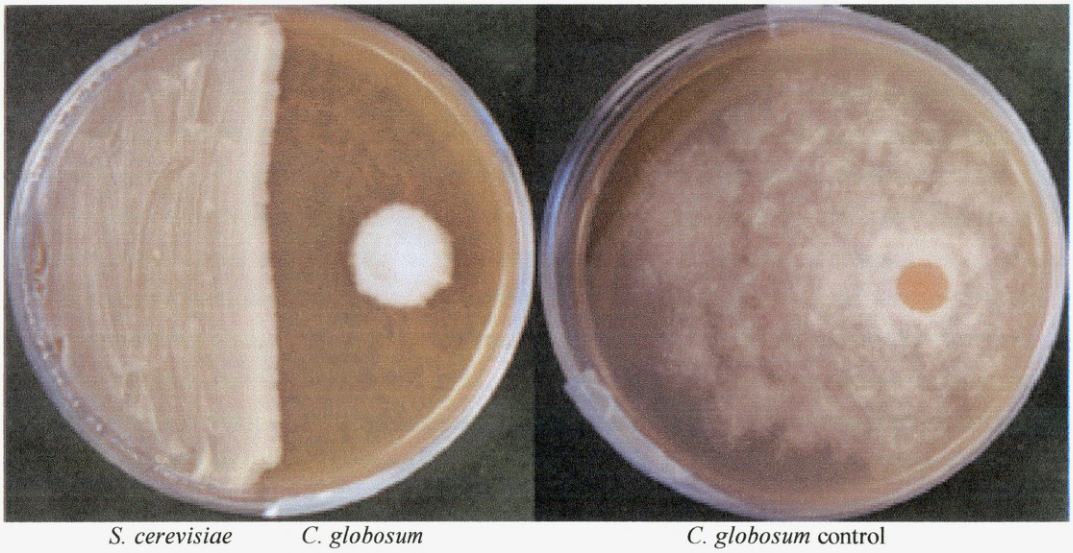
P. anomala *T. versicolor* *T. versicolor* control

Figure 3.6 Agar plate diffusion bioassay of *P. anomala NCYC 750* versus *T. versicolor*.



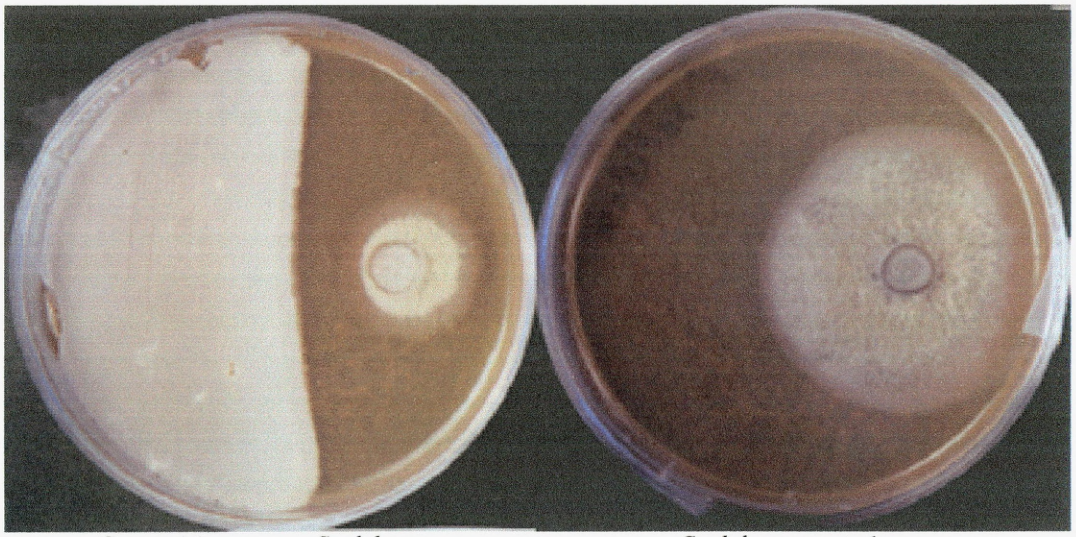
S. cerevisiae *T. versicolor* *T. versicolor* control

Figure 3.7 Agar diffusion bioassay of *S. cerevisiae* K28 versus *T. versicolor.*



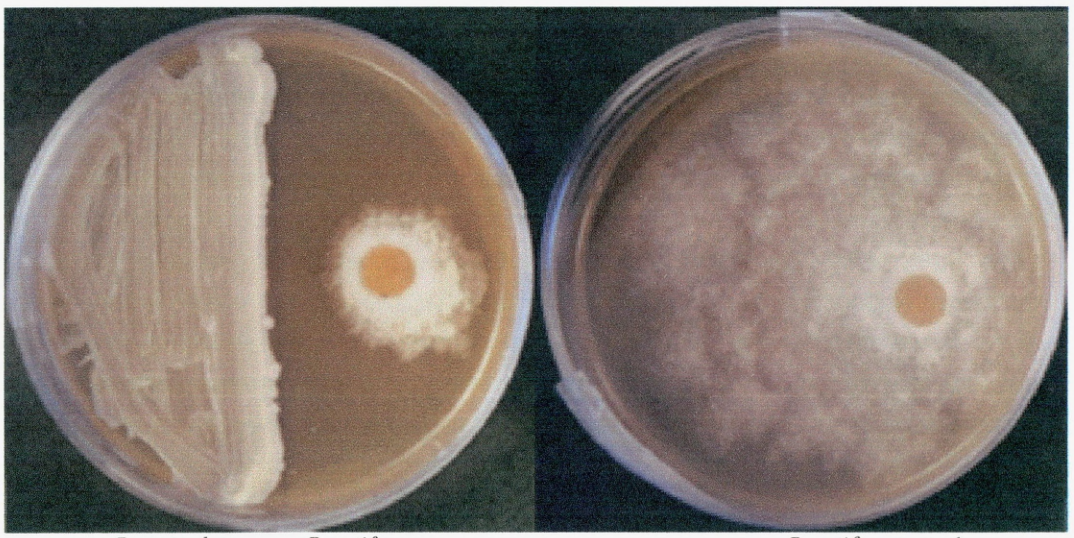
S. cerevisiae *C. globosum* *C. globosum* control

Figure 3.8 Agar diffusion bioassay of *S. cerevisiae* S 381 versus *C. globosum.*



S. cerevisiae *C. globosum* *C. globosum* control

Figure 3.9 Agar diffusion bioassay of *S. cerevisiae* K28 versus *C. globosum.*



P.anomala *P. setifera* *P. setifera* control

Figure 3.10 Agar diffusion bioassay of *P. anomala* NCYC 434 versus *P. setifera.*

3.4 **Discussion**

The results of the initial screens clearly showed that yeasts do affect fungal growth and that some yeasts are antagonistic to fungi to varying extents. The photographic data presented shows that in some cases this antagonism could lead to the almost complete cessation of fungal growth for at least two weeks. When all of the selected yeast strains were screened it became apparent that species from the *Saccharomyces* and *Pichia* genera were the most effective fungistatic yeasts. All of the yeasts used in this study have previously been described as killer yeasts, (Young and Yagiu 1978; Schmitt and Tipper 1990; Hodgson *et al.* 1995 and personal communications) and were confirmed to be killer strains previously (Section 2.1 and 2.4), with the exception of *S. cerevisiae* NCYC 1006 which has been recorded as being a sensitive yeast (Philliskirk and Young 1975). However, *S. cerevisiae* NCYC 1006 may be a killer yeast which has yet to be identified through the screening of a particular yeast sensitive to its killer toxin.

The pH measurements recorded during these assays showed a maximum pH drop between fungi and yeast of 0.5. This shows that a simple chemical alteration of the pH caused by compounds secreted by the yeast during growth such as acetic acid may be responsible for some of the growth inhibition observed. However this small drop in pH would not explain the strong inhibition seen in some of the assays as illustrated Figures 3.8, 3.9 and 3.10.

This initial screen was performed in order to identify yeast fungal antagonistic relationships for further study. Therefore, two yeasts identified in these initial screens as antagonistic to a number of different fungi and belonging to the *Saccharomyces* and

Pichia genera were selected for further study. These yeasts were *S. cerevisiae* strain K28 and *P. anomala* strain NCYC 750. *S. cerevisiae* strain K28 although not the most effective of the *S cerevisiae* strains screened was chosen due to its unusual and well documented killer activity (see section 1.221).

Three fungi were also selected for further study along with the above yeast strains namely, *C. globosum, P. placenta* and *T. versicolor.* These fungi were selected due to the strong inhibitory effect exhibited by the two selected yeasts on their growth and the fact that these three fungi are all strong biodeteriogenic agents of wood and their biocontrol would be of great use in wood preservation. These three fungi are also representatives of the three main wood decay types, as mentioned in section 1.14, with *C. globosum* being a soft rot fungi, *P. placenta* being a brown rot fungi and *T. versicolor* being a white rot fungi.

Chapter 4

Screening of yeast volatile metabolites for antifungal activity

4.1 Introduction

Yeasts are known to secrete volatile chemicals such as ethanol, aldehydes, esters etc. during their fermentative growth. These volatile chemicals may diffuse through agar, as in the previous bioassay and may be responsible for the inhibitory effects on fungal growth observed in the initial screening for yeast antifungal activity (Chapter 3) or volatile chemicals may diffuse through the air. Volatile chemicals produced by fungi from *Trichoderma* species have been reported to effect fungal growth in this manner (Dennis and Webster 1971a; Bruce *et al.* 1984; Bruce *et al.* 1996; Wheatley *et al.* 1997). Antifungal volatile chemicals have also been observed to be involved in the inhibition of fungal growth by bacteria (Fiddaman and Rossall 1993; Fernando and Linderman 1995). Therefore, an assay was performed to identify any effects on fungal growth caused by volatile chemicals secreted by the two yeast selected for further study, *S. cerevisiae* strain K28 and *P. anomala* strain NCYC 750. Three fungi were used for this assay, namely: *C. globosum, T. versicolor* and *P. placenta.*

4.2 Experimental Approach

The method used for this volatile assay was the method developed by Dennis and Webster (Dennis and Webster 1971a) for the screening of potential fungal biocontrol agents of plant pathogenic fungi and is described in Section 2.6.

4.3 Results

The results for these assays are displayed graphically in Figures 4.1 and 4.2. Figure 4.1 shows the effects of volatile chemicals secreted by the yeasts *S. cerevisiae* K28 and *P. anomala* NCYC 750 growing on complex media (malt extract agar 5%/2% (Section 2.21)) on fungal colony extension rate. Figure 4.2 shows the effects of volatile chemicals produced by yeast growing on the minimal media EMM 3 (Section 2.24) on the colony extension rate of the same fungi.

The effects of volatile chemicals secreted by yeast can be seen in these figures to be quite dramatic. This is especially so in the cases of *S. cerevisiae* K28 grown on complex media versus the two fungi *C. globosum* and *P. placenta* in which fungal growth is almost completely prevented. These same volatile secretions also affected the growth of *T. versicolor* although not as dramatically, with some fungal growth occurring after 3 days.

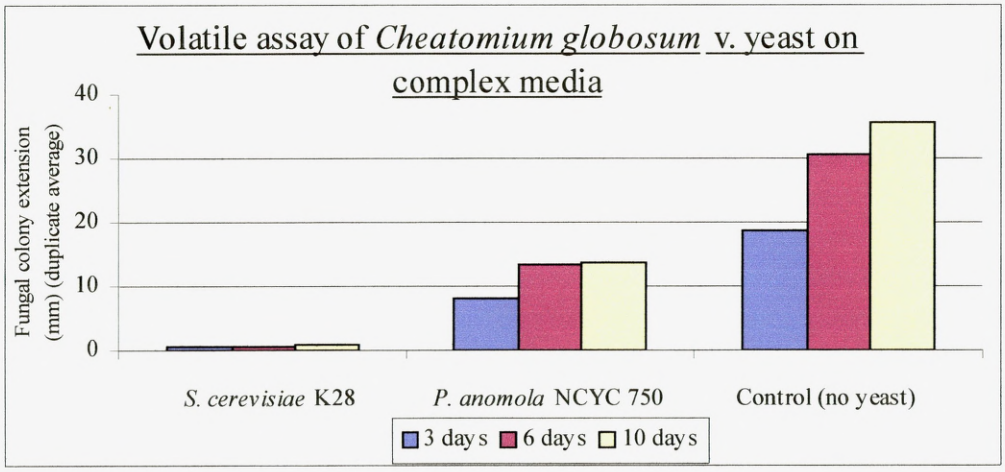
Although not as effective as *S. cerevisiae* K28, the volatile chemicals secreted by *P. anomala* NCYC 750 also affected fungal growth when grown on complex media. These volatile secretions inhibited fungal colony radial extension when compared to the control, by over 50% in the case of *P. anomala* NCYC 750 versus *C. globosum* and appeared to be more effective over time. This pattern of growth inhibition was also apparent in the case of *P. anomala* NCYC 750 versus *P. placenta* but was less evident versus *T. versicolor*.

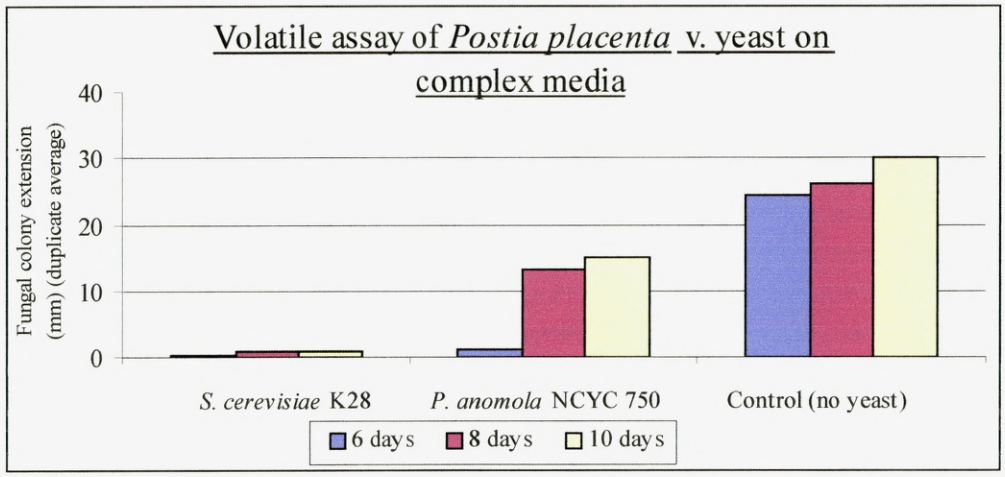
When grown on minimal media the effects of volatile chemical secretions by *S. cerevisiae* K28, although still evident, appeared to be less inhibitory to fungal growth than secretions from yeast grown on complex media. This was only true in the cases of *S.*

cerevisiae K28 versus *C. globosum* and *P. placenta*. In the assay using *T. versicolor* the effect of volatile secretions appeared to be the same whether the yeast *S. cerevisiae* K28 was grown on complex or minimal media.

This similarity in inhibition independent of media type was also observed for volatile chemical secretions by *P. anomala* NCYC 750 when grown on minimal media. The effects of these volatiles appeared to be just as potent as those secreted by the yeast when grown on complex media.

Figure 4.1 Influence of volatile chemicals, produced by yeast grown on complex media, on the colony extension rate of various fungi





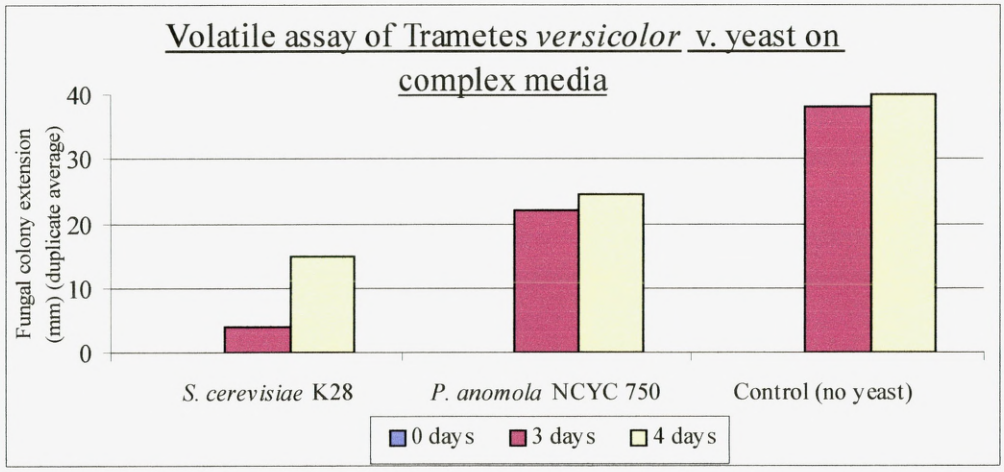
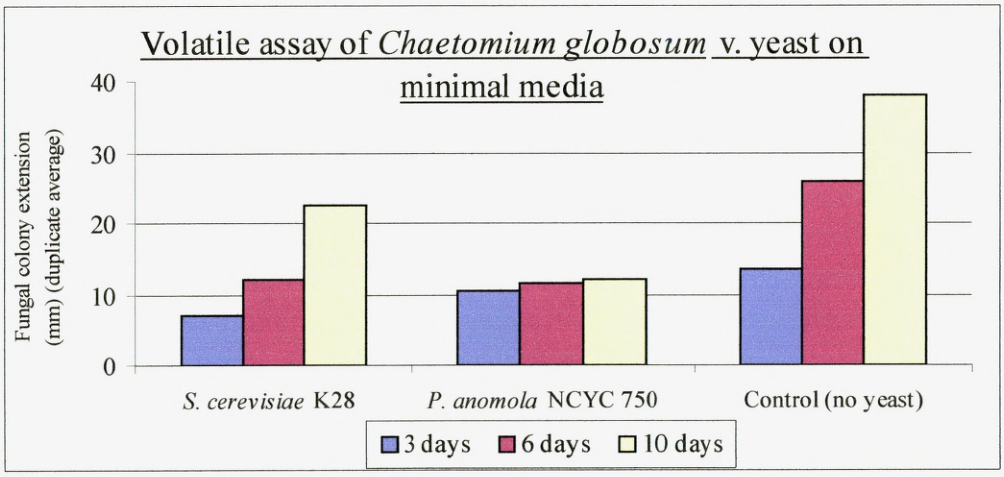
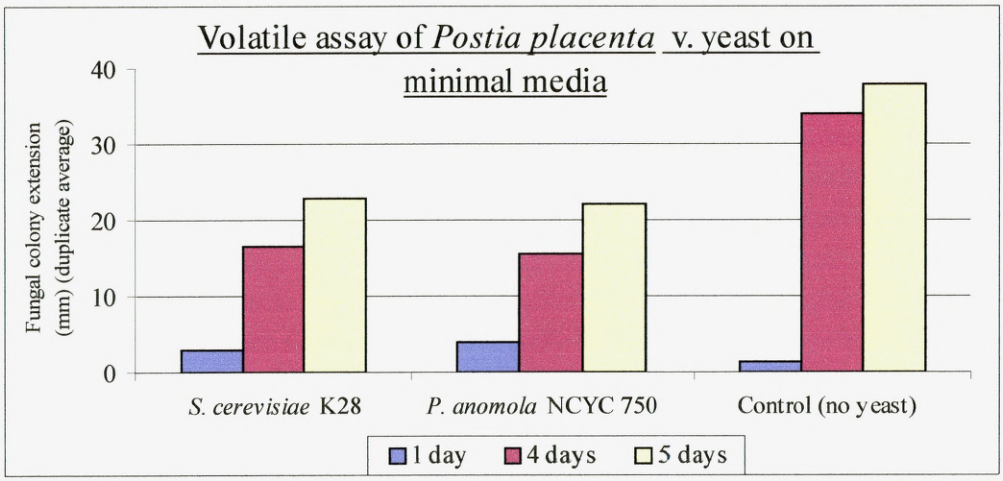
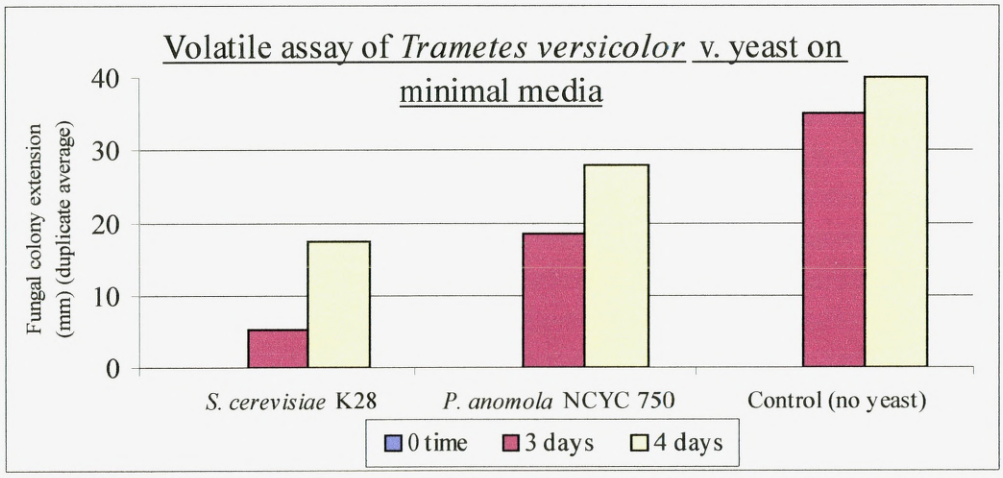


Figure 4.2 Influence of volatile chemicals, produced by yeast grown on minimal media, on the colony extension rate of various fungi







4.4 Discussion

The volatile chemicals secreted by yeast during growth can be effective inhibitors of fungal growth. This was seen to be so for both yeasts assayed but was especially apparent in the case of *S. cerevisiae* K28. This increased effectiveness may be due to either, the presence of a greater number of yeast cells on the *S. cerevisiae* K28 assays than on the P. anomala NCYC 750 assays or, to the fermentative properties of the *Saccharomyces* yeast species. This would result in the production of ethanol or higher alcohols which are known growth inhibitors of many microorganisms and can be sterilising agents in high enough quantities. This theory is reinforced by the decrease in fungal growth inhibition observed when minimal media was used as the yeast growth media. This minimal media contained less fermentable sugars and as such *S. cerevisiae* K28 would have secreted lower concentrations of ethanol or other higher alcohols resulting in less fungal inhibition. The inhibition of growth observed in the assays against *T. versicolor* was possibly due to volatile chemicals other than primary fermentation products such as CO_2, acids (eg. acetic acid or keto acids) or aldehydes and the production of these volatiles must be unchanged in response to media composition.

P. anomala is not as vigorous a fermentative yeast as *S. cerevisiae* and therefore although ethanol would have been secreted by *P. anomala* NCYC 750 the concentration of this production would be less than in the *S. cerevisiae* K28 assay. However the assays of *P. anomala* NCYC 750 did show inhibition of fungal growth when compared with the no yeast control assay. Also, as in the case of *S. cerevisiae* K28 versus *T. versicolor,* the assays of *P. anomala* NCYC 750 did not show large differences in inhibition when the yeast was grown on complex or minimal media. As such some other volatile chemicals

other than fermentation products may be at work here and these volatile chemicals must be produced independently of media composition. These volatile chemicals may be normal products of metabolic growth such as carbon dioxide, acetic acid or ethyl acetate or could be the products of specific anabolic pathways yet to be identified in yeast.

Up to seventy two volatile products have been identified from fungi of *Trichoderma* spp. (Bruce *et al.* 1996; Wheatley *et al.* 1997) at least some of which exhibit antifungal properties (Humphris *et al.* 2001), alkyl pyrones have been identified as an antifungal volatile secreted by *T. harzianum* (Claydon *et al.* 1987) and numerous compounds including 2-methyl-propanol, 1-octen-3-ol, 3-octanone, 3-methylfuran and ethyl acetate have been identified as common volatile secretions of *Aspergillus, Fusarium* and *Penicillium* in cereal feeds (Schnürer *et al.* 1999). Ethyl acetate has also recently been suggested as a putative antifungal volatile secreted by *P. anomala* (Fredlund *et al.* 2001). In bacteria, ammonia has been identified as a potential volatile responsible for fungal inhibition (Howell *et al.* 1988; Fernando and Linderman 1995; Ejechi 1998), however, yeast are not commonly found to secrete ammonia in any large quantity and thus this is unlikely to be the case here. The production and/or secretion of such products may be a normal signaling process analogous to the pheromone secretions of mammals or the antimicrobial secretions of actinomycetes or could be in response to the presence of the fungi being controlled.

Chapter 5

Production and assays of putative antifungal agents from yeasts

5.1 Introduction

Previous studies of the antagonistic affects of yeast against fungi have suggested many modes of action for the antifungal activity observed. One of these modes of action could be the secretion of an antifungal compound or "mycocin". Some studies have found evidence for such a secreted antimycotic agent (Yamasaki *et al.* 1951) but in most cases of yeast versus fungi little data has been evident to support this theory. As discussed previously killer toxins secreted by yeasts, as well as affecting the growth of other yeasts (Bevan and Makower 1963; Rogers and Bevan 1978; Kazantseva and Zimina 1989; Buzzini and Martini 2000b) and bacteria (Polonelli and Morace 1986; Morace *et al.* 1989; Izgu and Tinbay 1997), exhibit some antifungal activity (Polonelli *et al.* 1987; Walker *et al.* 1995).

The previous chapter demonstrated that volatile chemicals secreted by yeast can also dramatically affect fungal growth. However, a secreted mycocin, possibly similar to or even identical to a yeast killer toxin, may also be partly responsible for the inhibition of fungal growth observed in the initial screens (see Chapter 3). Such mycocins may be secreted in low concentrations and as such, concentration of spent growth media may aid in the characterisation of any mycocins secreted by yeast.

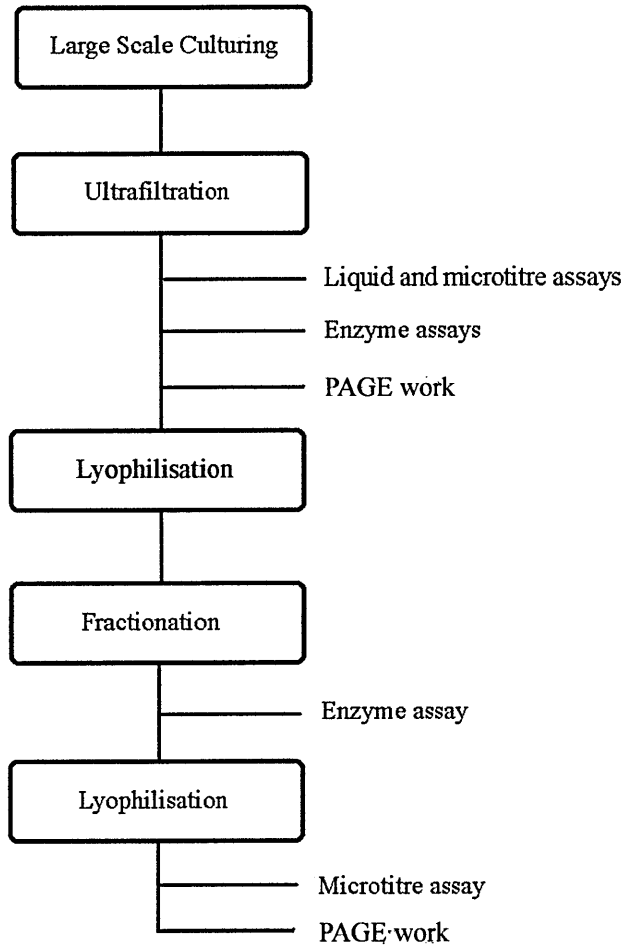
Therefore, the two yeasts identified as being of interest in this study, in terms of their antifungal activity, namely *S. cerevisiae* strain K28 and *P. anomala* strain NCYC

750 were cultured in larger volumes and the spent growth media was concentrated for further study as described below.

5.2 **Experimental Approach**

The yeast strains S. cerevisiae K28 and *P. anomala* NCYC 750 were cultured, purified and assayed for antifungal activity as described below and illustrated in Figure 5.1. Each step of this process will be described, and any results shown, in a stepwise fashion as illustrated in Figure 5.1. A discussion of all of the findings will be presented at the end of the chapter.

Figure 5.1 Methodological process of putative mycocin production and purification



5.3 Large scale production of putative mycocin

The putative mycocin was prepared using a method similar to those described in previous studies of yeast killer toxins (Middelbeck *et al.* 1979; Pfeiffer and Radler 1982; McCracken *et al.* 1994; Hodgson *et al.* 1995) as described below.

Each yeast was grown in EMM3 media in a 20 litre LH fermentor (LH series 1075, Inceltech, Reading, Berks.) at 25°C and with an agitation speed of 100rpm. Growth was stopped after 48 hrs by removal of cells in order to avoid the build up of lysed cell products within the media. Cell harvesting was performed using a continuous centrifuge (Heraeus Christ, Contifuge 17 RS, Brentford, Essex) at 10000 rpm (14500 g) and a pump rate of 30ml/min. The resulting supernatant was then sterile filtered using a 0.2 μm filtration membrane in order to remove any remaining yeast cells.

The cell free extract obtained after filtration was then ultrafiltered through a 10,000 MW cutoff membrane in a Millipore Pellicon cassette system ultrafiltration unit (Millipore, Watford, Hearts). This ultrafiltration was continued until approximately 1L retentate remained thus facilitating the approximate 20 fold concentration of any macromolecules present in the now cell free extract with an approximate mass above 10 kDa. The retentate from this ultrafiltration was stored in 100ml amounts at -20°C prior to use in a liquid assay (see below) to assess any antifungal activity.

5.4 **Assays of concentrated cell free supernatant**

5.41 Liquid assay of concentrated yeast supernatant

The partially concentrated cell free extracts from *S. cerevisiae* strain K28 and *P. anomala* strain NCYC 750 were assayed for antifungal activity through the use of a liquid assay described below. The fungi used were the same fungi used in the previous volatile assay (Chapter 4), namely *C. globosum, T. versicolor* and *P. placenta.*

Yeast and fungal stocks were prepared as in the two previous assays (descried in Sections 2.5 and 2.6). The assays were performed in 100ml conical flasks containing 45 ml of malt extract broth (Oxoid). The volumes of each flask were then made up to 50 ml by the addition of one of the following treatments:

A) 5ml of an actively growing 24 hr malt extract broth yeast culture containing approx 10^7 cells /ml.

B) 5 ml of partially concentrated yeast supernatant

C) 5 ml of partially concentrated yeast supernatant which had been heated to 100° C for 10 minutes.

D) 5 ml of sterile distilled water.

A 4mm fungal core taken from an actively growing fungal culture was then placed in each flask to grow as a pellicle.

All flasks were set up in duplicate and were then incubated at 23°C for two weeks. At the end of this time fungal growth was measured through dry weight measurements as follows.

The fungal mycelia from each flask were separated from the growth media and majority of yeast cells by filtration through a coarse filter made with a pre weighed piece of triple folded muslin. The fungi and muslin were then dried at 98°C for 24 hours and weighed. Two pieces of triple folded muslin were used to filter sterile malt extract broth and were also dried at 98°C for 24 hours. The weight loss of these pieces was used to calculate the percentage weight loss of the muslin during the drying process. The recorded weights of the pre weighed muslin pieces used for the dry weight measurements of fungi were then adjusted using this percentage.

Fungal mass was calculated by subtraction of the adjusted pre weighed muslin weight from the dried weight, as is shown in the following formula.

$$\text{Dry wt fungi} = X2 - \left(X \times \frac{Y1}{Y} \right)$$

Where X = wt muslin

X2 = Dry wt muslin + fungi

Y = wt muslin used for standardisation

Y1 = dry wt muslin used for standardisation

5.411 **Results of the liquid assay of concentrated yeast supernatant**

The results are shown graphically in Figures 5.2 and 5.3 below and are illustrated photographically in Figure 5.4.

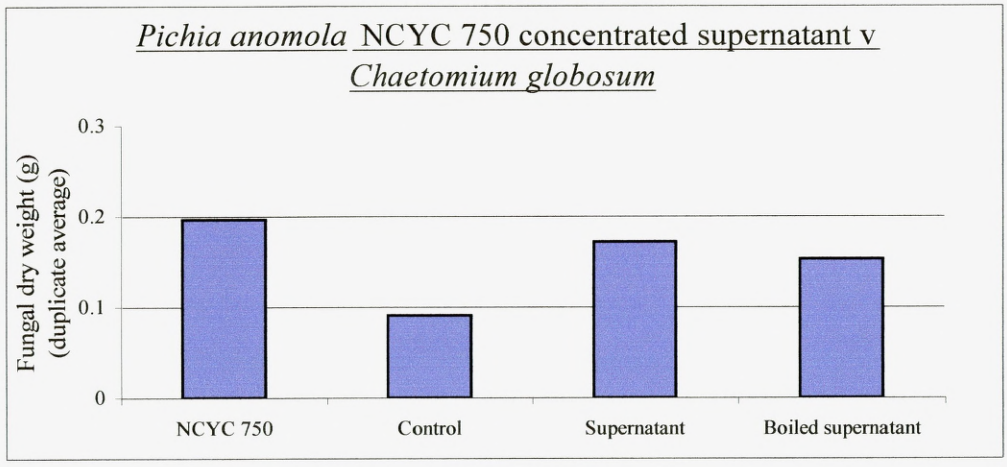
The assays performed using *P. anomala* NCYC 750 partially concentrated supernatant showed no decrease in fungal dry weight measurement when any of the three fungi were assayed. In fact, an increase in dry weight measurements of 89% of the control dry weight for *C. globosum,* 40% for *P. placenta* and 21% for *T. versicolor* was observed. Boiling of the ultrafiltrate decreased this effect in the case of *C. globosum* with an increased dry weight of 69% and virtually eliminated the effect for *P. placenta* and *T. versicolor* with dry weights of 16% more and 8% less than the control, respectively. Increases in fungal dry weight (in comparison with controls) also occurred when live yeast where grown with the fungi except in the case of *P. placenta* where a 72% drop in fungal dry weight was observed.

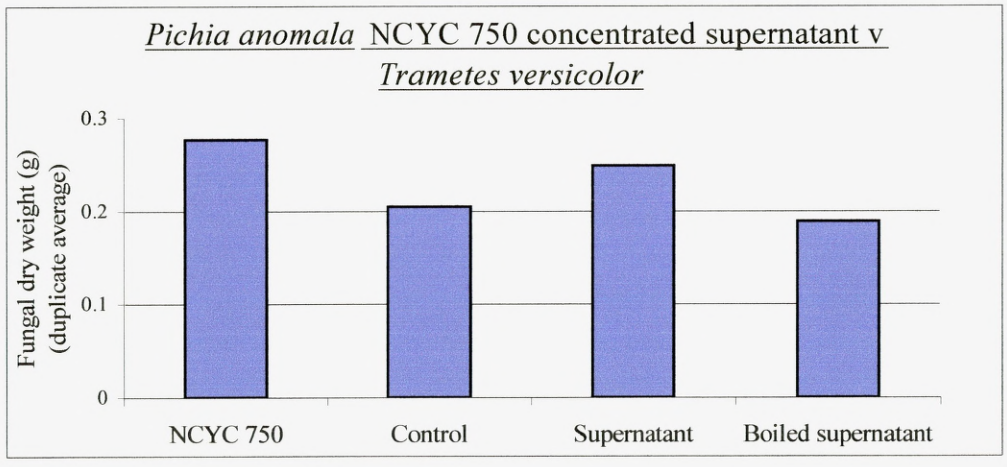
In assays of *C. globosum* and *T. versicolor* the *S. cerevisiae* K28 partially concentrated supernatant had very little effect on fungal dry weight with no change and 4% more weight than the controls observed, respectively. The boiled concentrated yeast supernatant samples also had little effect on the dry weights of these fungi with 6.5% more weight than the control recorded for *C. globosum* and 8% less weight than the control recorded for *T. versicolor*. However, in the assays of *P. placenta* the partially concentrated yeast supernatant caused a decrease in fungal dry weight of 30% when compared to the control and the boiled extract increased the dry weight recorded by 48%. When each of the fungi were grown with live yeast, a decrease in dry weight

measurement was recorded of 79%, 65% and 60% for *P. placenta, T. versicolor* and *C. globosum,* respectively.

When the fungal growth in the flasks was viewed, however, the effects of the putative mycocins in the cell free extracts become apparent. In Figure 5.4 the photographs of the assay of *S. cerevisiae* K28 versus *C. globosum* a difference in fungal growth morphology was observed with the fungi growing not as a normal mycelial mat but in stringy and clumped masses.

Figure 5.2 Liquid assays of partially concentrated *P. anomala* NCYC 750 supernatant against various fungi.





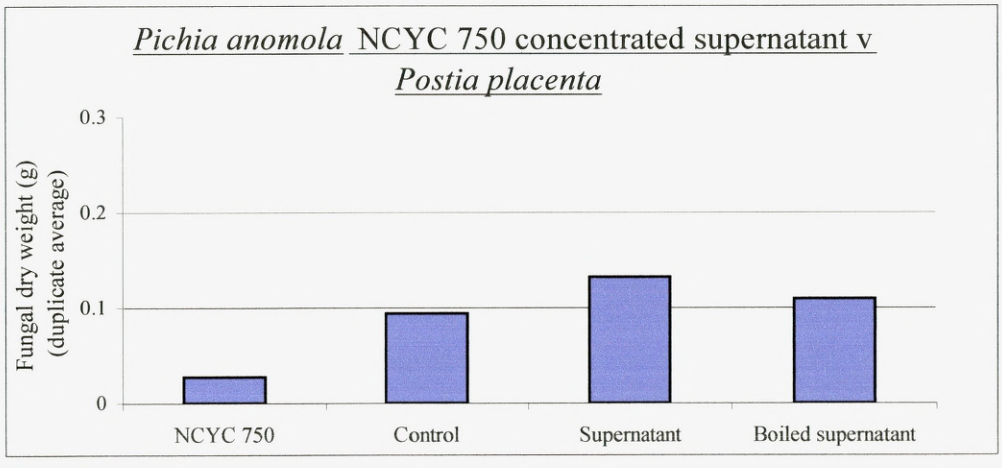
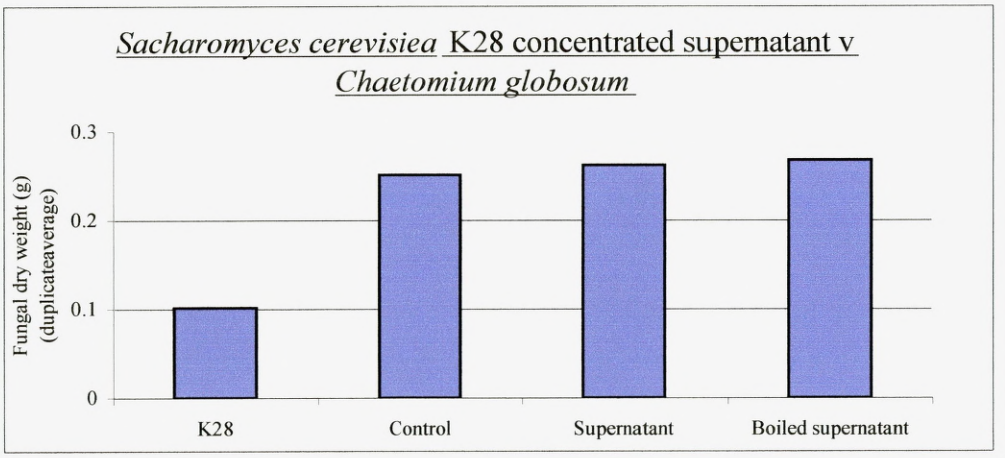
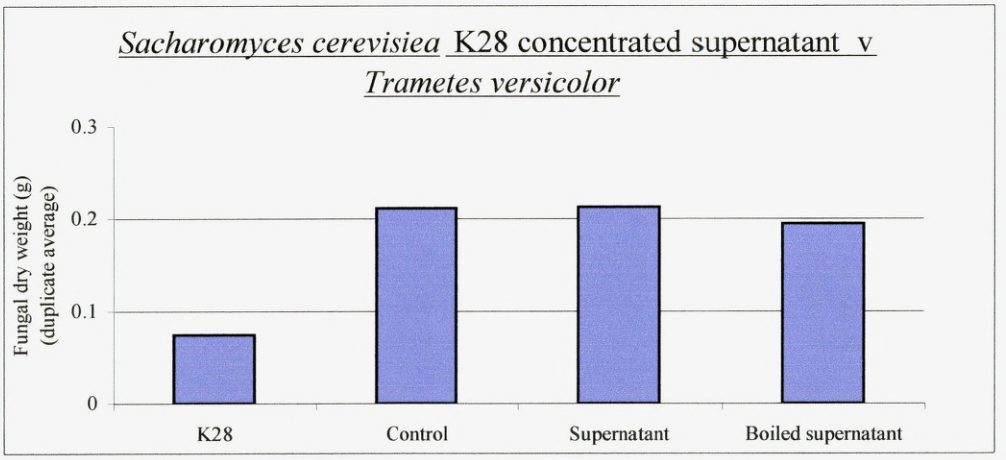


Figure 5.3 Liquid assay of partially concentrated *S. cerevisiae* K28 supernatant against various fungi.





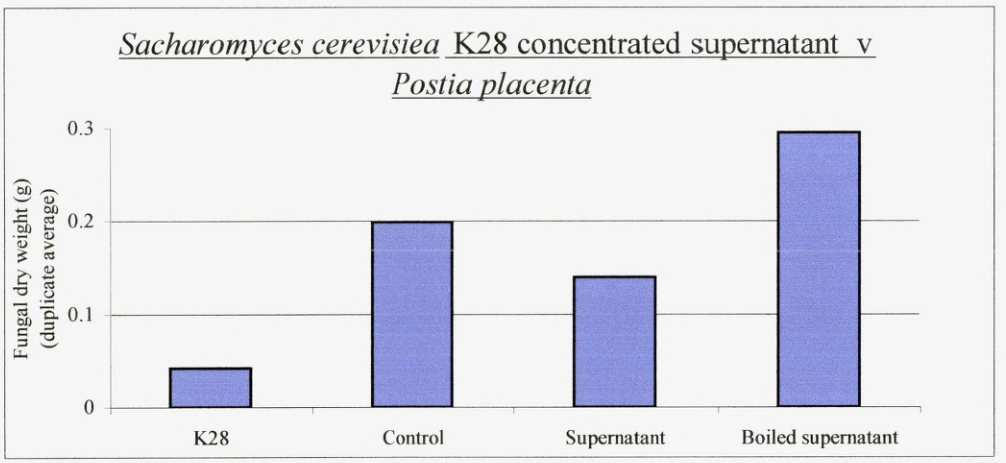
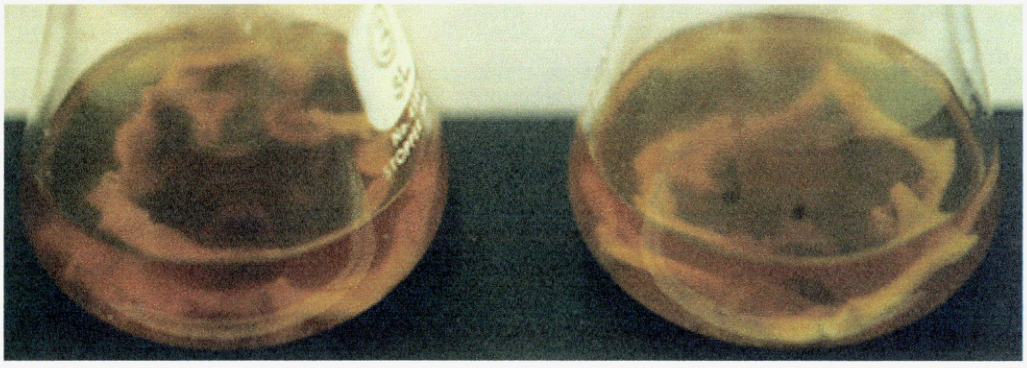
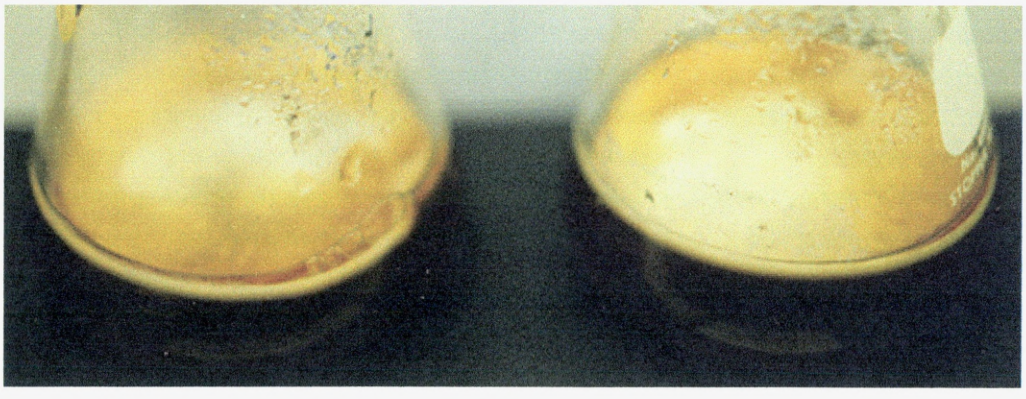


Figure 5.4 Photographs illustrating the effect of *S. cerevisiae* cell free supernatant on the growth of *C. globosum.*



Liquid culture of *C. globosum* in malt extract broth containing the concentrated supernatant of *S. cerevisiae.*



Liquid culture of *C. globosum* in malt extract broth. (Control)

5.42 **Microtitre assay of partially purified supernatant from *S. cerevisiae* K28 and *P. anomala* NCYC 750 for antifungal activity.**

A microtitre assay using fungal mycelial fragments was developed for this and subsequent assays from a method by Broekaert *et. al.* (Broekaert *et al.* 1990). Due to the limited amount of concentrated cell free supernatant obtained for the previous assay (Section 5.2) this assay was performed with only one fungus namely, *C. globosum.*

The fungus was grown in a liquid culture of malt extract broth under no agitation at 22°C for 5 to 7 days. An approximately 1cm square piece of the mycelial mat was then removed and macerated using a Potter homogeniser with a 1mm gap between plunger and tube. The resulting mycelial fragment mixture was then centrifuged and the spent malt extract broth removed. The mycelial fragments were then resuspended, to an approximate concentration of 2000 fragments per ml, in fresh double strength malt extract broth. Aliquots of 100µl of this mixture were then placed in the wells of a microtitre plate.

The concentrated yeast supernatant containing any putative antifungal agent was diluted using citrate buffer (Section 2.32) by factors of 3:1, 1:1 and 1:3 giving percentage supernatant concentrations of 75, 50 and 25. Aliquots of 100µl of each of these concentrations plus the 100% partially concentrated toxin were added to the inoculated microtitre wells.

The following control treatments were used:

1) 200µl of malt extract broth containing no fungal fragments
2) 100µl of malt extract broth with fragments plus 100µl of malt extract broth containing no fungal fragments

3) 100µl of malt extract broth with fragments plus 100µl of 100% concentrated yeast supernatant which had boiled for 10 mins.

All assays mixtures were inoculated in triplicate and the microtitre plates were immediately analysed using an automated microtitre plate reader (Dynatech MR5000, Dynatech Laboratories. Ltd, UK) in order to obtain a baseline reading for each well. Readings were taken after a 10 second agitation period in the microtitre plate reader and were measured at an absorbance of 630 nm.

Further readings were taken at least once per day and the baseline reading was subtracted from these readings in order to standardize the assay results.

5.421 **Results of the microtitre assays of partially purified yeast supernatants.**

The results for the above assays are displayed in Figures 5.5 and 5.6 and show the fungal growth inhibition caused by the partially concentrated cell free supernatants from *P. anomala* NCYC 750 and *S. cerevisiae* K28.

Figure 5.5 shows the assay of *S. cerevisiae* K28 partially concentrated yeast supernatant versus the wood decay fungus *C. globosum.* In this assay fungal growth, measured by absorption at 630 nm, was almost entirely inhibited for 90 hours by the 100% concentrated yeast supernatant. Growth was also completely inhibited for the first 48 hours of the assay by the 75% dilution of the concentrated yeast supernatant, and was only 28% of the control after 90 hours. The 50% and 25% diluted concentrated yeast supernatant inhibited growth to 88% and 36% after 48 hours and 52% and 0% after 90 hours, respectively, when compared to the control's growth in malt extract broth.

The results were similar in the case of *P. anomala* NCYC 750 as displayed in Figure 5.6 with fungal growth when exposed to the 100% concentrated yeast supernatant recorded as 11% of the control after 48 hours and 18% after 90 hours. The fungus when exposed to the 75% 50% and 25% dilutions of the yeast supernatant showed 12%, 50% and 70% of the control growth after 48 hours and 36%, 94% and 126%, respectively, after 90 hours.

In both cases boiling had little effect on the potency of the antifungal properties of the concentrated yeast supernatant. The boiled *S. cerevisiae* K28 concentrated yeast supernatant caused fungal growth of 0% of the control after 48 hours and 10% after 90 hours whilst fungal growth with the boiled *P. anomala* NCYC 750 concentrated yeast supernatant was 4% after 48 hours and 21% after 90 hours.

Figure 5.5 Graph showing effect of decreasing concentration of *S. cerevisiae* K28 supernatant versus *C. globosum* in a microtitre assay.

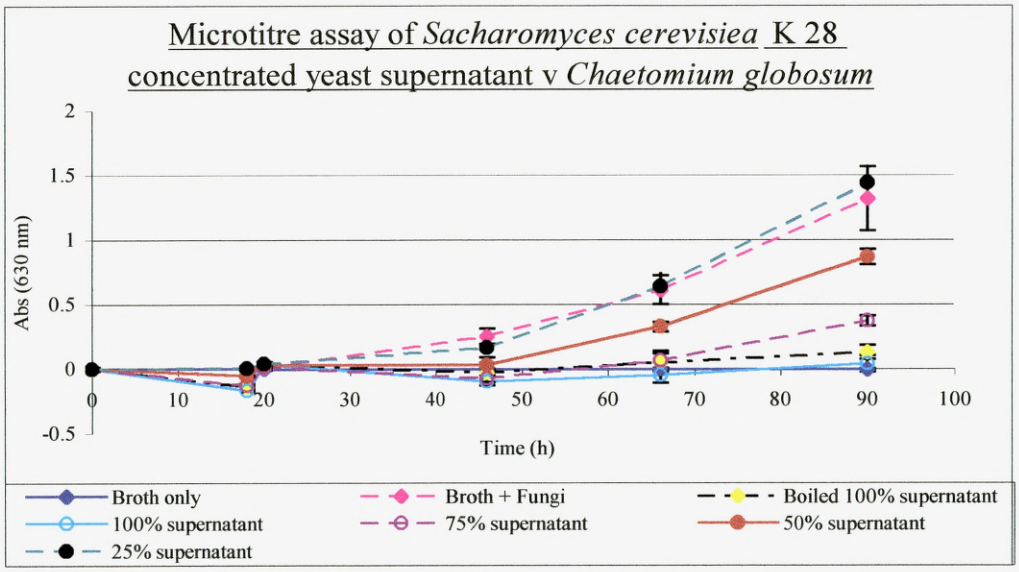
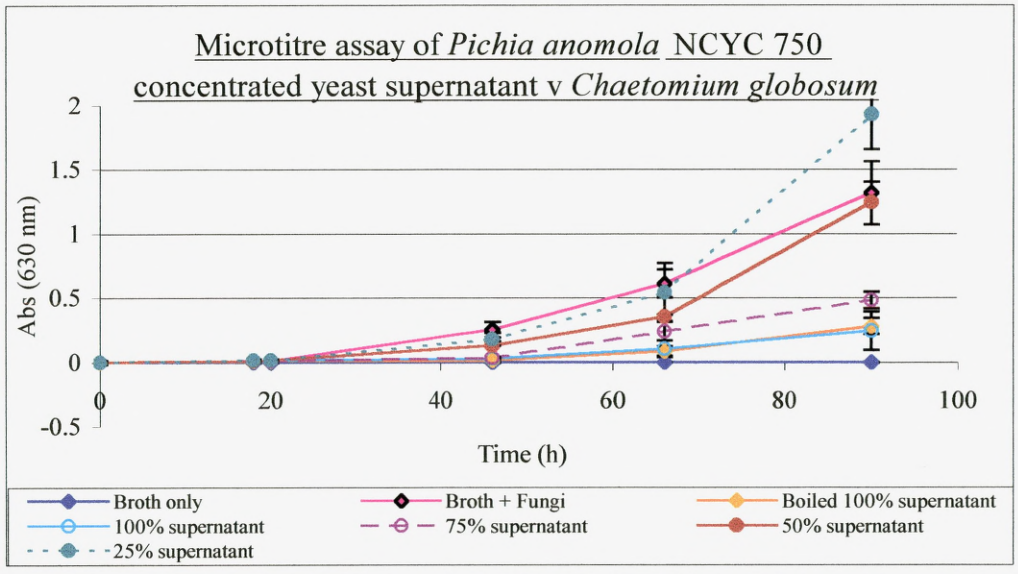


Figure 5.6 Graph showing effect of decreasing concentration of *P. anomala* NCYC 750 supernatant versus *C. globosum* in a microtitre assay.



(The error bars displayed on the graphs in Figure 5.5 and Figure 5.6 show the sample standard deviation of the triplicate results obtained for each data point.)

5.43 **Enzyme Assays**

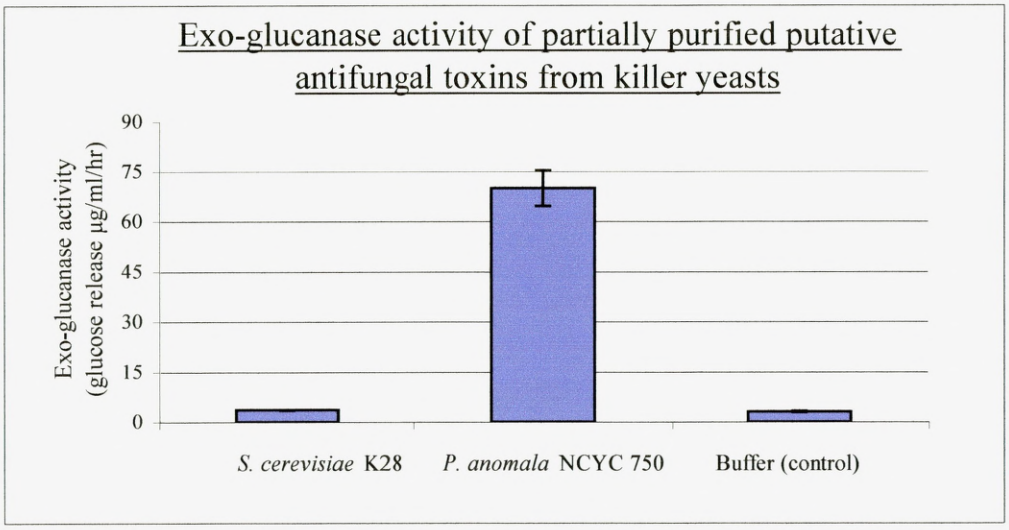
Results reported above and in chapters 3 and 4 of this thesis have shown that secreted products from the yeasts *S. cerevisiae* K28 and *P. anomala* NCYC 750 do possess antifungal properties. The mechanism by which these antifungal properties are mediated, however, has yet to be identified. The mycocin may be or be related to a yeast killer toxin and as such is probably proteinaceous in nature and may possess some enzymatic activities, which may be partly or entirely responsible for the antifungal activities observed. Two enzymes known to be involved in the biocontrol of fungi by some fungal, yeast and bacterial biological control agents are chitinase and exo-glucanase (laminarinase) (Wisniewski *et al.* 1991; Lorito *et al.* 1993; Bruce *et al.* 1995; Castoria *et al.* 1997; Grevesse *et al.* 1998a; Grevesse *et al.* 1998b; Jijakli and Lepoivre 1998; Lee and Kim 1999; Gomes *et al.* 2001) due to the fact that chitin and glucans are major components of fungal cell walls. Therefore the cell free ultrafiltered extracts (which may be described henceforth as partially purified toxins) from *S. cerevisiae* K28 and *P. anomala* NCYC 750 were tested for chitinase and exo-glucanase activity using the enzyme assays described in Chapter 2 (Section 2.7).

5.431 **Results of enzyme assays**

Exo-glucanase

The results from the exo-glucanase assay are shown in Figure 5.7. These show that the partially purified toxin from *P. anomala* NCYC 750 did exhibit some exo-glucanase activity with a glucose release of 78 µmols/ml/hr. The partially purified toxin from *S. cerevisiae* K28, however, showed no activity.

Figure 5.7 Exo-β-1,3-glucanase activity of partially concentrated crude yeast mycocins.



The error bars shown on the graph are for the standard deviations in the data, which were 0.14, 6.61 and 0.25 from left to right on the graph

Chitinase

Chitinase activity could not be detected in cell free ultrafiltrates from either *S. cerevisiae* K28 or *P. anomala* NCYC 750.

Due to the fact that no enzymatic activity was observed in the concentrated cell free supernatant of *S. cerevisiae* K28 all further work was performed using the cell free concentrated supernatant obtained from *P. anomala* NCYC 750.

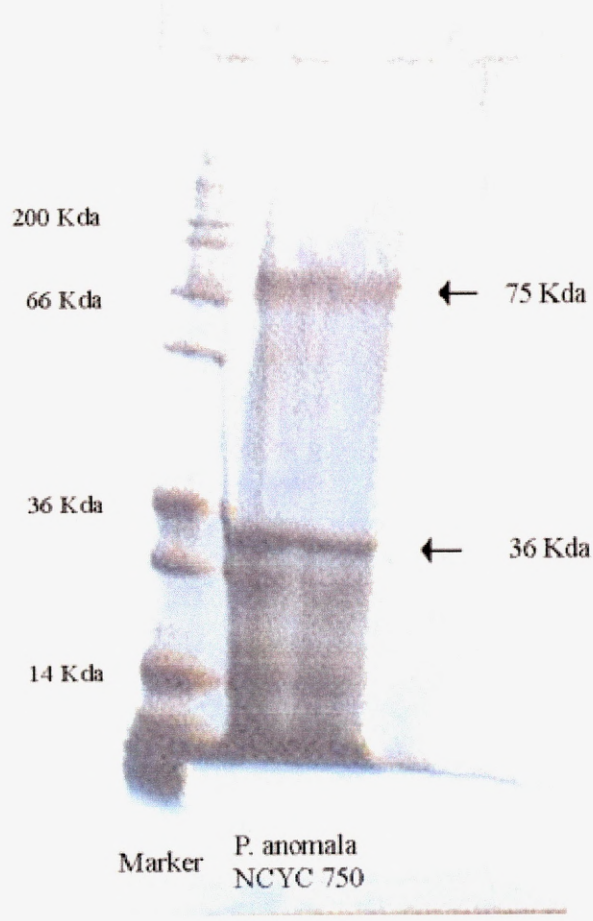
5.5 **SDS-PAGE gel analysis of concentrated putative mycocin**

The cell free extract of *P. anomala* NCYC 750 was analysed using SDS-PAGE in order to determine the approximate size of the any secreted proteinaceous putative antifungal agents. SDS-PAGE was performed using the conventional SDS-PAGE method described in Section 2.81. *P. anomala* NCYC 750 fractions were concentrated tenfold by lyophilisation and resuspension in a one tenth volume of citrate buffer in order to enhance the appearance of any proteins on the gel. Staining of the completed gel was performed using the silver staining procedure described in Section 2.83.

5.51 **Results of SDS-PAGE gels**

The silver stained completed gel obtained from the SDS-PAGE analysis of concentrated *P. anomala* NCYC 750 cell free supernatant is shown in Figure 5.8. The Gel clearly shows the presence of a number of proteins in the concentrated cell free extract. The resolution of the protein bands contained in the *P. anomala* NCYC 750 fraction was not clear after staining and any protein bands below approximately 30 kDa appear to blur together. However two bands are relatively clear and these appear to represent proteins with masses of approximately 36 kDa and 75 kDa.

Figure 5.8 SDS-PAGE gel of cell free concentrated *P. anomala* NCYC 750 supernatant



5.6 Fractionation of concentrated cell free supernatant of *P. anomala* NCYC 750

Yeast killer toxins vary greatly in size and form and are generally proteinaceous in nature being either proteins or glycoproteins (see Chapter1). The secreted yeast "mycocins" from *P. anomala* NCYC 750 is probably a proteinaceous compound, as it appears to exhibit enzymatic activity and is of at least 10 kDa in size as it was retained after the ultrafiltration process undertaken during the "mycocin" production and concentration (Section 5.3). However the compounds could be much larger than this 10 kDa and therefore, a mechanism by which the concentrated cell free extracts could be fractionated by molecular mass was used to gain further insight into the macromolecular nature of these compounds. This process also allowed for the further purification and concentration of the presumed mycocins.

5.61 Experimental Approach

Fractionation was undertaken using a Fast Performance Liquid Chromatography (FPLC) separation technique. A BioRad FPLC system was used with a BioRad fraction collector (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK). The column used was a Superdex 75 HR 10/30 Column (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK) containing dextran covalently bonded to highly cross-linked agarose beads. The column was cleaned prior to use as per the BioRad instruction manual.

Concentrated *P. anomala* NCYC 750 cell free supernatant samples were prepared by lyophilisation of 10ml quantities and resuspension of the freeze dried product in 1ml of citrate buffer at pH 4.5.

The column was used with a citrate buffer eluent as the yeast samples were suspended in this buffer (Chapter 2). A buffer flow rate through the column of 5ml per min was set and buffer was passed through the column for at least one hour prior to use in order to obtain a steady baseline reading. The 10 fold concentrated partially purified yeast toxins were loaded onto the column in volumes of 200µl and the flow rate was adjusted to 0.5ml per min. Fractions were collected using a BioRad automated fraction collector set for 2 minute (1ml) intervals and collection was undertaken for at least 70 minutes (35 x 1ml fractions). A detector measuring absorbance at 280nm was used to detect proteins eluted from the column and a chart recorder running at 2mm per min and measuring a full scale deflection of an absorbance of 1.0 was used to record the fractions containing protein.

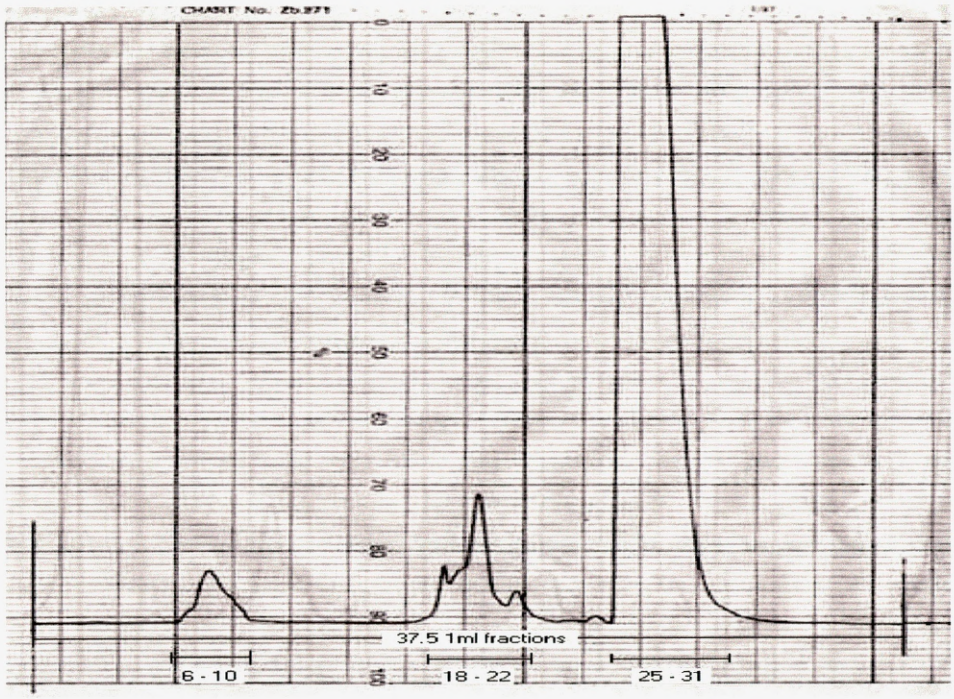
5.62 Results of FPLC fractionation of concentrated cell free supernatant of *P. anomala* NCYC 750 toxin

The results of the FPLC analysis, and fractionation of the putative antifungal agents(s) from *P. anomala* NCYC 750, are displayed in Figure 5.9 which shows the absorbance pattern at 280nm of eluent from the Superdex 75 HR 10/30 Column over a 70 minute run.

Three clusters of absorption peaks were seen on the FPLC absorbance trace. The first peak occurred after 12 minutes (6ml of eluent) and ended after 20 mins (10ml of

eluent). This was the lowest complex of absorption peaks with a maximum absorption of approx. 0.07. The second peak occurred after 36 minutes (18ml of eluent) and ended after 44 minutes (22ml of eluent). This complex of peaks in absorption appeared to be a collection of at least 3 peaks with maximum absorption's of 0.07, 0.18 and 0.05. The final peak was recorded after 50 minutes (25ml of eluent) and ended after 62 mins (31 ml of eluent). This peak was off scale for most of this 12 minute period and therefore may have been a collection of a number of peaks.

Figure 5.9 Absorption profile at 280nm of eluent from *P. anomala* NCYC 750 cell free supernatant after a single run through an FPLC column.



5.7 Assays of Fractionated *P. anomala* NCYC 750 cell free supernatant

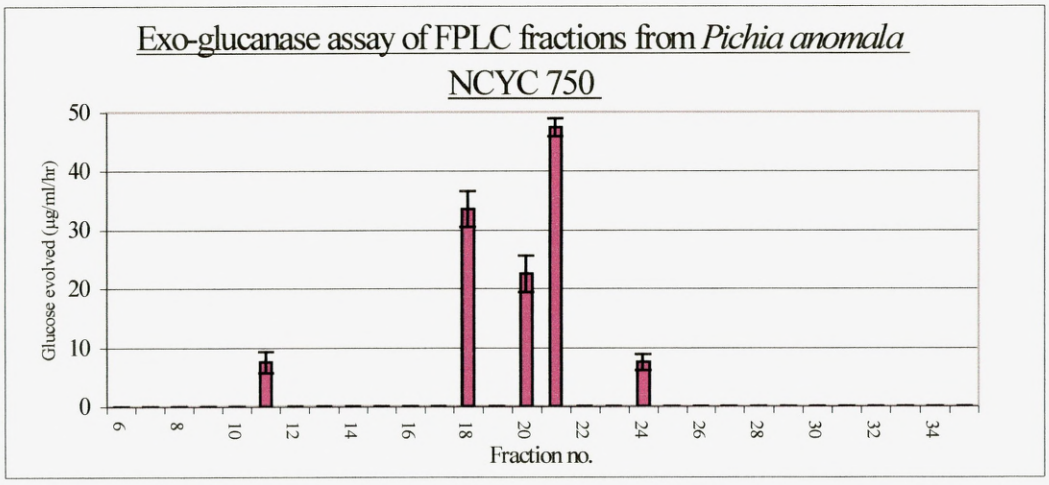
5.71 Exo-glucanase assay of *P. anomala* NCYC 750 fractions

As reported in Section 5.431 exo-glucanase activity was detected in the partially concentrated *P. anomala* NCYC 750 cell free extract. Accordingly, twenty nine of the NCYC 750 fractions (fractions 6 to 35), obtained from the FPLC fractionation of *P. anomala* NCYC 750 concentrated cell free supernatant (Section 5.6), were tested for exo-glucanase activity in order to determine which fraction or fractions exhibited this enzyme activity and to identify the approximate size of the enzymes responsible for this activity. The exo-glucanase assay described previously (Section 2.71) was used again for this assay and all sample assays were performed in triplicate.

5.711 Results of exo-glucanase assay of FPLC fractions from the *P. anomala* NCYC 750

The results of this assay, shown in Figure 5.10, clearly show the presence of exo-glucanase activity in five of the twenty nine samples tested. Samples eleven and twenty four showed very low exo-glucanase activity of 7.5 μg/ml/hr of glucose evolved. The other three samples testing positive, namely fractions eighteen, twenty and twenty one showed higher exo-glucanase activities of 33.25, 22.5 and 48.75 μg/ml/hr of glucose evolution, respectively. All three of these fractions exhibiting higher exo-glucanase activity came from the second peak on the FPLC trace. Error bars show standard deviation for each sample.

Figure 5.10 Exo-glucanase activity of *P. anomala* NCYC 750 fractions after FPLC fractionation.



Error bars show standard deviation for each sample.

5.72 **Microtitre assay of pooled concentrated NCYC 750 fractions**

The previous experiments, described in Sections 5.6 and 5.71, demonstrated that the partially purified putative antifungal agent from killer yeasts *P. anomala* NCYC 750 gave three collections of peaks observed on an FPLC absorption trace (Figure 5.9) after fractionation, namely fractions 6 to 10, 18 to 22 and 25 to 31. These three peaks were pooled together, for further assaying and analysis as described below, and will henceforth be referred to as fraction 1 (6 to 10), fraction 2 (18 to 22) and fraction 3 (25 to 31).

Further concentration of putative mycocins was performed by freeze drying the pooled fractions and resuspending the lyophilised product in a one tenth volume of citrate buffer (Section 2.32). A microtitre assay similar to the assay performed using the concentrated cell free supernatant (Section 5.42) was undertaken using the concentrated-pooled fractions (1 to 3) in order to test for any antifungal activity. The assays were performed against the three wood decay fungi used previously namely, *C. globosum, T. versicolor* and *P. placenta.*

Mycelial fragment preparation was undertaken as per the method described previously (Section 5.42) and the fragments were resuspended in malt extract broth to a concentration of 2000 fragments per ml. The mycelial fragments were dispensed in 100µl aliquots into wells on a microtitre plate. The following preparations and controls were then added to each well:

i. 100µl of fraction 1

ii. 100µl of fraction 2

iii. 100µl of fraction 3

iv. 100µl of partially concentrated unfractionated cell free extract

v. 100µl of sterile water

vi. 100µl of citrate buffer

vii. 100µl of malt extract

An eighth well was also inoculated with 100µl of double strength malt extract containing no fragment and 100µl of citrate buffer, as a final control.

All assay mixtures were inoculated in triplicate. The microtitre plates were immediately analysed using an automated microtitre plate reader (Dynatech MR5000) in order to obtain a baseline reading for each well. The readings were taken after a 10 second agitation period in the microtitre plate reader and were measured at an absorbance of 630 nm. Further readings were taken at least once per day and the baseline reading was subtracted from these readings in order to standardise the results.

5.721 **Results of microtitre assays for antifungal activity in FPLC fractions from *P. anomala* NCYC 750**

All three assays showed inhibition of fungal growth as can be seen in Figures 5.11, 5.12 and 5.13. The degree of inhibition and the effect of the different fractions however varied greatly between the three fungi tested. Standard deviations were calculated for each point of each data set and are displayed as error bars on the graphs (Figures 5.11, 5.12 and 5.13). The standard deviations in the results increased over the duration of the assay and after 144 hours this error was so great that no statistically meaningful results could be obtained.

In the assay of the fungus *C. globosum*, the three *P. anomala* NCYC 750 fractions showed very similar effects for the first 72 hours, with fungal growth being 38% of the growth observed for the malt extract control for fraction 1, 35% for fraction 2 and 22% for fraction 3. This pattern could also be seen when growth is compared alongside the water control with fungal growth equaling 51% (fraction 1), 48% (fraction 2) and 30% (fraction3) of the controls growth being recorded. The buffer control though showed a slightly different pattern with growth in fractions 1 and 2 being comparable to the control and growth in fraction 3 being 62% of the control.

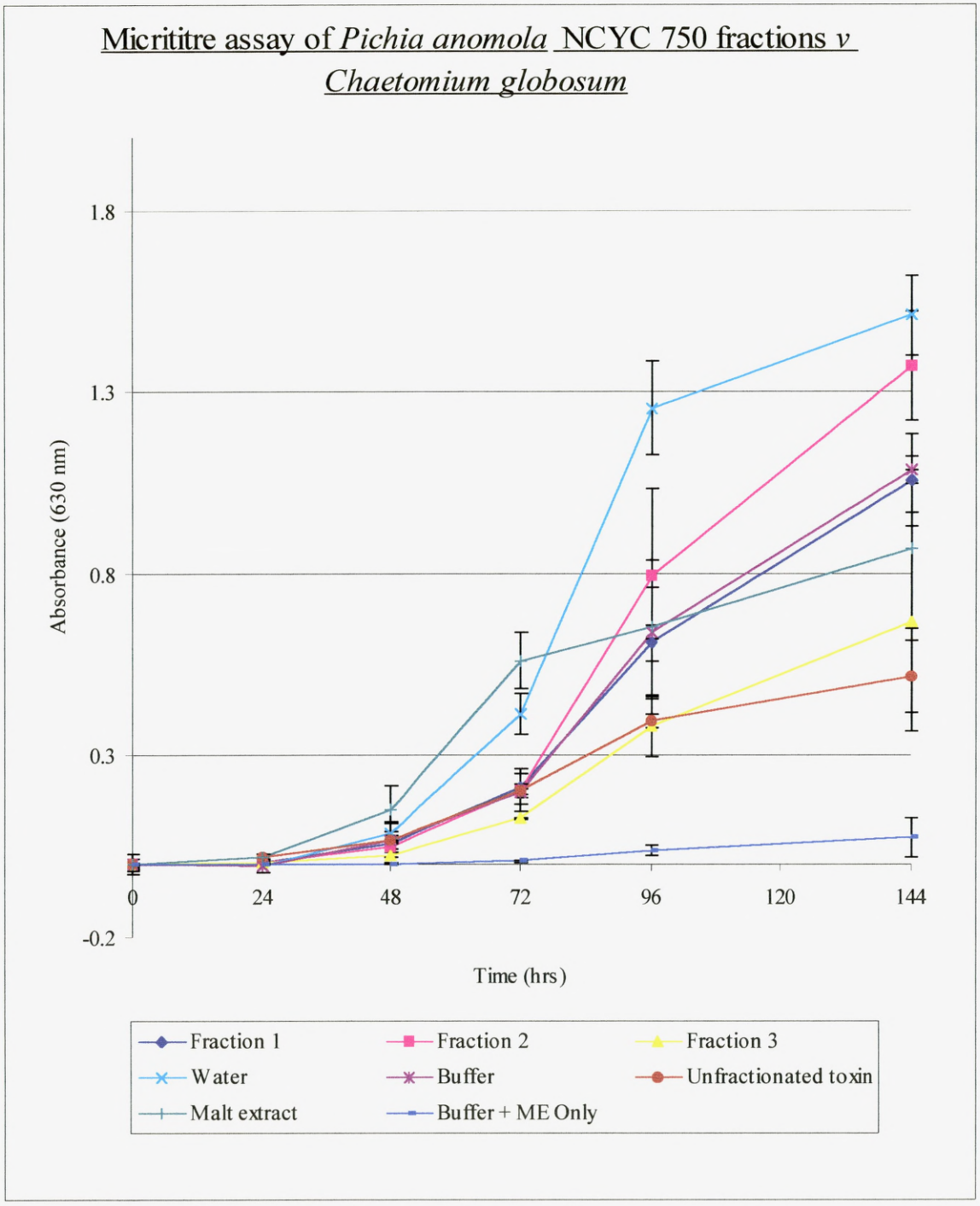
This pattern changed after 144 hours with fungal growth being similar or greater for fractions 1 and 2 when compared with the malt extract and buffer controls. Fraction 3 still caused less growth in these cases at 76% of growth in malt extract and 61% of buffer. Fungal growth when compared to the water control after 144 hours was again lower with fraction 2 showing 90% growth, fraction 1 showing 69% growth and fraction 3 showing 44% growth. The unfractionated partially purified, unfractionated cell free extract of *P. anomala* NCYC 750 showed approximately 10% less growth than that observed for fraction 3 in each case.

T. versicolor also showed the least amount of growth in the presence of *P. anomala* NCYC 750 fraction 3. This growth was around 5% of each control after 72 hours and between 3 and 6 % after 144 hours. However, the fungus also displayed decreased growth in the presence of fraction 2 and fraction 1. This was most pronounced in the presence of fraction 2 with approximately 10% of all of the controls' growth observed after 72 hours and 13, 16 and 22% of the malt extract, citrate buffer and water controls' respectively, growth observed after 144 hours. For fraction 1, growth was

approximately 50% of all controls (slightly less for the water) after 72 hours and 24% of the malt extract, 31% of the citrate buffer and 42% of the water controls' after 144 hours. Fungal growth in the presence of the unfractionated toxin was greater than all three of the controls after 72 and 144 hours.

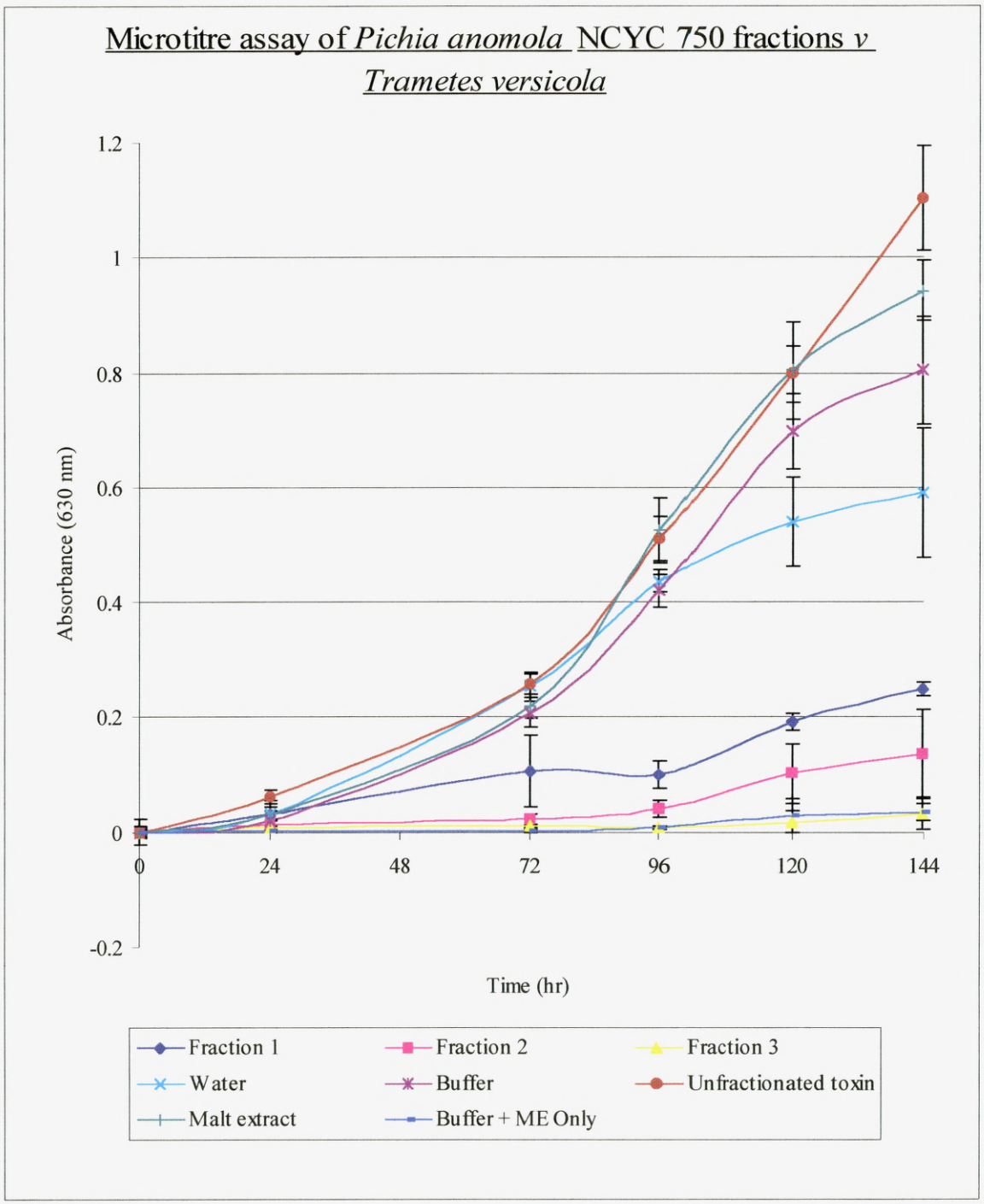
The figures for the growth of *P. placenta* in the presence of the various fractions also showed the least growth of the fungi in the presence of fraction 3. This growth was approximately 4% of all controls after 72 hours and 1% or less after 144 hours. Growth in the presence of fraction 2 again showed some decrease in the first 72 hours (between 20 and 30%) and an even more pronounced decrease after 144 hours of 14% of the malt extract control and 6.5% of the water and buffer controls. Fraction one showed little effect after 72 hours (slightly less growth than the malt extract control) but showed a dramatic decrease in growth after 144 hours of 5 % of the malt extract control and 2.3% of the water and buffer controls. In the presence of the unfractionated toxin the growth of *P. placenta* was greater than that of all three controls after 72 and 144 hours.

Figure 5.11 Graph showing results of a microtitre assay for antimycotic effects of *P. anomala* NCYC 750 secreted compounds against *C. globosum.*



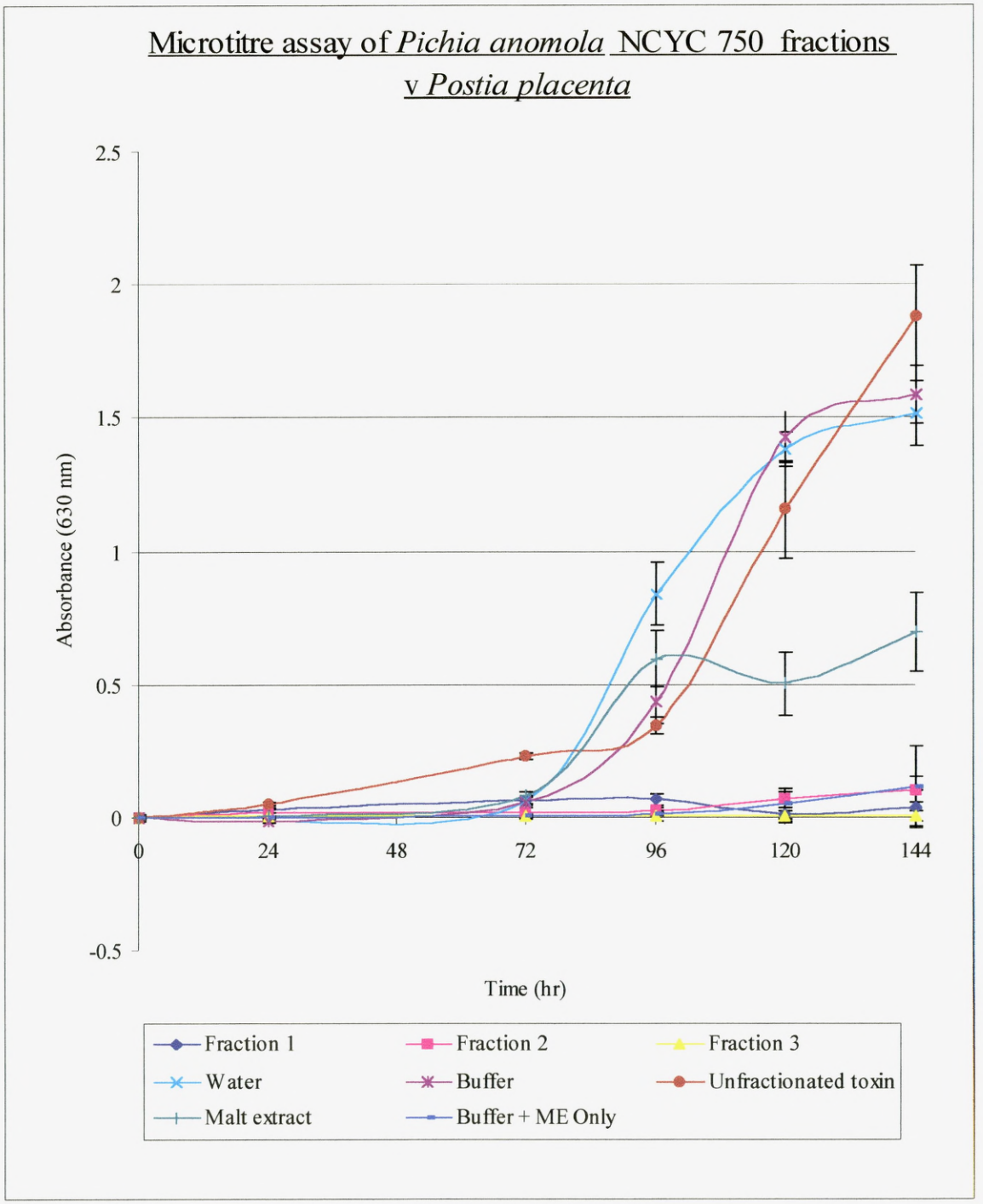
Error bars show standard deviation in triplicate samples

Figure 5.12 Graph showing results of a microtitre assay for antimycotic effects of *P. anomala* NCYC 750 secreted compounds against *T. versicolor*.



Error bars show standard deviation in triplicate samples

Figure 5.13 Graph showing results of a microtitre assay for antimycotic effects of *P. anomala* NCYC 750 secreted compounds against *P. placenta*.



Error bars show standard deviation in triplicate samples

5.8 Gel electrophoresis of fractionated *P. anomala* NCYC 750 cell free supernatant.

The pooled concentrated NCYC 750 fractions (1 to 3) and the unfractionated NCYC 750 toxin used in the previous assay were run on electrophoresis gels in order to determine the approximate sizes of the putative antifungal proteins present. A fifth sample was included containing tenfold concentrated (lyophilisation and resuspension in a one tenth volume) pooled fractions (the 12 to 13 minute fractions) representing a flat non protein containing elution from the FPLC fractionation. Gel electrophoresis was undertaken using a NuPage Bis Tris electrophoresis system as described in Section 2.82 and the completed gels were stained using the silver staining method described in Section 2.83. Protein gels were analysed using Phoretix gel analysis software in order to determine the protein band sizes exhibited.

5.81 Results of Gel electrophoresis of fractionated P. anomala NCYC 750 cell free supernatant.

The completed stained gel from the NuPAGE Bis Tris electrophoresis of the fractionated *P. anomala* NCYC 750 cell free supernatant is shown in Figure 5.14. The stained gel shows the presence of protein in the samples of sizes indicated by the markers and as described below:

The unfractionated toxin showed numerous protein bands four of which are illustrated in Figure 5.14. At least 3 feint bands were present around mark A (Figure 5.14) exhibiting molecular masses between 68 and 101 kDa and a fifth broad band was

present between the A and B marks representing a protein with an approximate molecular mass of 57 kDa.

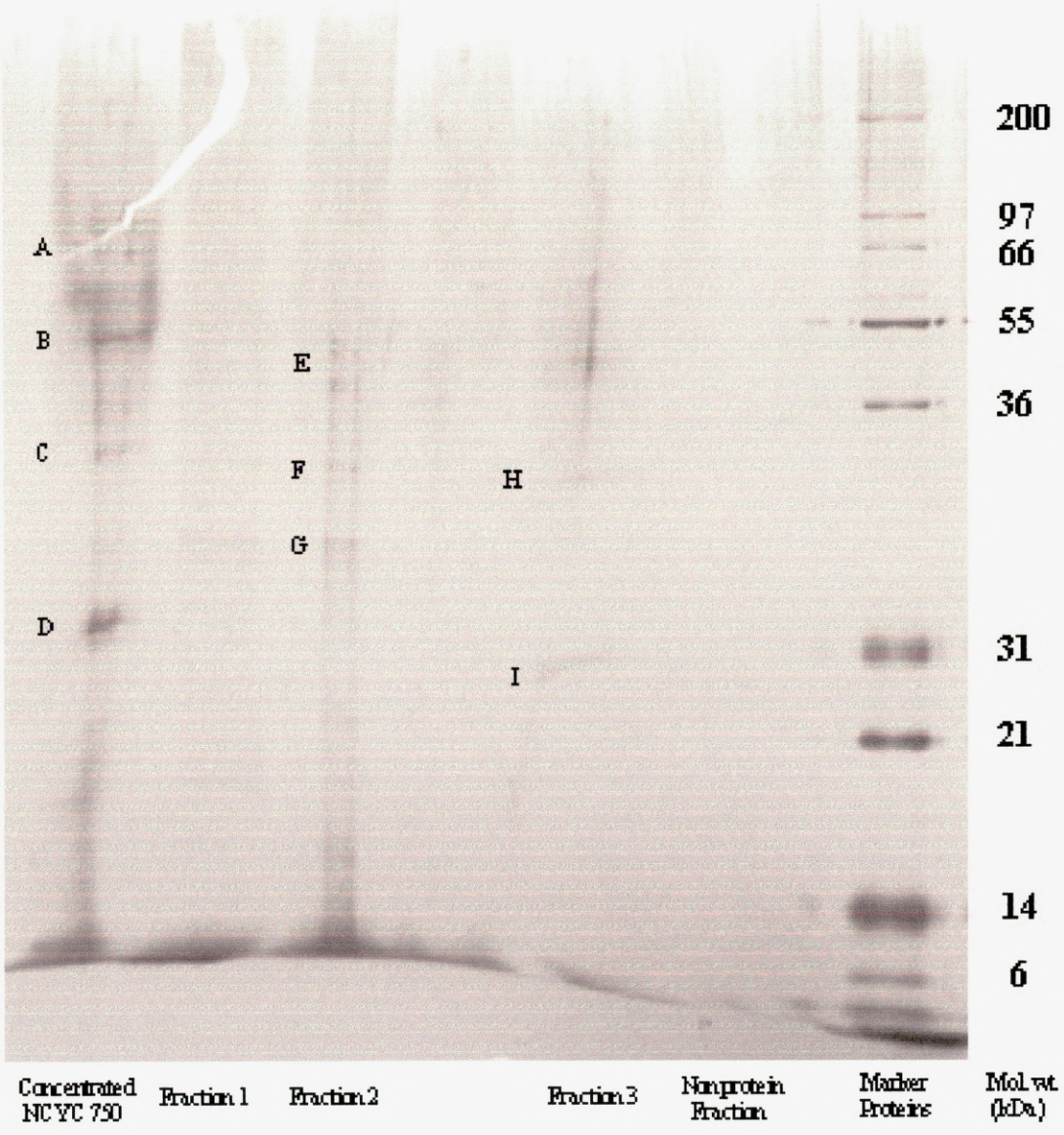
Fraction 1 showed no clear protein bands.

Fraction 2 showed numerous feint bands, the three clearest of which are picked out in Figure 5.14 and represent proteins with masses of 57, 26 and 23 kDa.

Fraction 3 showed at least three feint bands two of which are picked out in Figure 5.14. The third feint blurred band above mark H (Figure 5.14) could represent a protein with a molecular mass of approximately 39 kDa.

The non protein FPLC sample showed no protein bands.

Figure 5.14 Silver stained NuPAGE Bis Tris gel of fractionated *P. anomala* NCYC 750 cell free supernatant.



A to I mark band molecular sizes as follows:

A = 92 kDa	B = 45 kDa	C = 28 kDa
D = 20 kDa	E = 57 kDa	F = 26 kDa
G = 23 kDa	H = 26 kDa	I = 20 kDa

5.9 **Discussion**

The results from the liquid assays of the putative antifungal concentrated cell free supernatants from *S. cerevisiae* K28 and *P. anomala* NCYC 750 against the fungi *C. globosum, T. versicolor* and *P. placenta* illustrated in Figures 5.2 and 5.3 appear to show very little or no inhibition of fungal growth and in many cases what appears to be stimulation of growth. With the exception of the assay of the *S. cerevisiae* K28 concentrated yeast supernatant versus *P. placenta,* this lack of growth inhibition, exhibited by the two concentrated cell free supernatants, appears to be unaffected by the boiling of the supernatant. In the assay of *S. cerevisiae* K28 concentrated yeast supernatant versus *P. placenta* the yeast supernatant appeared to inhibit fungal growth whilst the boiled supernatant appeared to be deactivated and actually stimulated the growth of the fungi. This observed inhibition represented only a 30% drop in dry weight, and thus fungal biomass, which would not be considered high enough for the effective biocontrol of wood decay fungi. Biocontrol of wood decay, as discussed in Chapter 1 (Section 1.14), must be long term and in the case of structural materials must be close to 100% effective as a failure due to a loss of structural integrity in the wood could result in serious consequences. A 30 % reduction in fungal biomass, if associated with a 30% drop in decay may, however, be acceptable in some non structural cases where a lengthened lifespan of the wood may be beneficial.

This lack of inhibition was not observed with the *S. cerevisiae* K28 live yeast cells (Section 5.411 and Figure 5.3). In these assays the three fungi appeared to be greatly inhibited showing decreases in fungal growth as assessed by dry weights. This fungal growth inhibition caused by live yeast could be mediated by a number of factors such as;

competition between the fungi and yeast for the available nutrients in the liquid growth media, the ability of the yeast to grow rapidly deoxygenating the media. In light of the results displayed in Chapter 4 showing the effect of volatile chemical secretions by yeast on fungal growth, this inhibitory effect could have been due to the secretion of volatile chemicals especially ethanol and higher alcohols by the fermenting yeast or may be associated with high carbon dioxide levels in the supernatant which could also be inhibitory to fungal growth. This effect was not observed with the *P. anomala* NCYC 750 live yeast cells (Figure 5.2) suggesting that this yeast is not as effective as *S. cerevisiae* K28 at rapid sequestration of nutrients or that different chemicals, volatile and non volatile, are secreted by the two yeasts under study.

These liquid assays also illustrate a problem in assays of fungi, with an example of difficulties in the replicability of fungal growth (personal communication Dr Nia White, University of Abertay Dundee). The four assays involving *C. globosum* and *P. placenta* (top and bottom graphs in Figures 5.2 and 5.2) show differences in the growth observed of the control (fungi only) assay. The control fungal growth for *C. globosum* and *P. placenta*, recorded by dry weight, in the assay of partially concentrated *P. anomala* NCYC 750 supernatant was over 50% less than the growth observed in the assay of partially purified *S. cerevisiae* K28 supernatant. Both assays were performed under identical conditions and each was performed in duplicate. A greater number of replicates in these assays may balance out this apparent lack of replicability and may alter the findings discussed above.

Even though the majority of the liquid assays described above did not show any antifungal activity, measured in the form of fungal biomass decreases, and problems were

experienced in the replicability of fungal growth, the photographs from the assay of *S. cerevisiae* K28 versus *C. globosum* do show an effect caused by the yeast supernatant in the form of differences in fungal morphology. These differences were almost certainly caused by the presence of a putative antifungal agent in the yeast supernatant of *S. cerevisiae* K28 and therefore, even though differences in fungal biomass were not observed in all cases, some form of antagonism may have been taking place in all of the assays. The lack of observed inhibition may have been due to low concentrations of the antifungal agents in the yeast supernatant assayed, and therefore the strength of any antagonism occurring, may not have been enough to show any changes in biomass or physiology in the other assays. As well as this any antagonism is unlikely to have been due to the presence of volatile chemicals as any volatile present should have been lost during the concentration of the partially purified supernatants.

As such, the presence of secreted antifungal agents was considered to still be possible and the use of a different, more sensitive method of assessing possible fungal growth inhibition by the putative antifungal agents of *S. cerevisiae* K28 and *P. anomala* NCYC 750 was undertaken.

This second assay of the cell free extract (Section 5.42), which was designed to measure growth inhibition through differences in fungal morphology, showed significant fungal growth inhibition. This inhibition was 100% over 4 days in the case of the *S. cerevisiae* K28 toxin and was over 80% after 4 days with *P. anomala* NCYC 750 toxin. The inhibitory effect was also concentration dependent as demonstrated by a step wise decrease in intensity following dilution of the toxin showing that the inhibition was almost certainly due to an antifungal compound present in the concentrated cell free yeast

supernatants. These agents could be yeast killer toxins as these are known to be secreted into growth media and would be concentrated by the size of ultrafiltration membrane used. Yeast killer toxins vary in size from 10 to over 100 kDa (Golubev 1998) and the killer toxin of *S. cerevisiae* K28 has been purified and characterised to be a heterodimeric glycoprotein with subunits of 10.5 and 11 kDa (Schmitt and Tipper 1995). Killer toxins from strains of *Pichia* have also been purified and characterised to be glycoproteins of weights 19 kDa for *P. kluyveri*, (Middelbeck *et al.* 1979), 25 kDa for *P. farinosa*, (Suzuki and Nikkuni 1994) and 83.3 kDa for *P. anomala*, (Sawant *et al.* 1989). However the antifungal activity of the agents present in these two concentrates was not diminished to any great extent by boiling, and yeast killer toxins being proteinaceous in nature would ordinarily be expected to be destroyed by high temperature (Middelbeck *et al.* 1979; Suzuki and Nikkuni 1989; Radler *et al.* 1993; McCracken *et al.* 1994; Hodgson *et al.* 1995; Kono and Himeno 1996). This does not preclude the possibility that yeast killer toxins are responsible for the antifungal activity observed, as heat stable toxins have been reported in the yeasts *W. mrakii* (Ashida *et al.* 1983), *Ustilago maydis* (Kandel and Koltin 1978). Nevertheless, this evidence suggests that hitherto unreported heat stable secreted compounds from known killer yeast strains exhibit antifungal activity and point to the potential practical application of such agents against wood decay fungi.

The enzyme assays (Section 5.43) demonstrated the possibility that the mycocin responsible for the antifungal activity caused by *P. anomala* NCYC 750 may be enzymatic in nature and exhibits some exo-glucanase activity and the SDS-PAGE gel work (Section 5.5) showed the presence of proteins, one of which could be the antimycotic enzymes, with masses of approximately 75 kDa, 36 kDa and less than 30

kDa. Previous work undertaken by Yamamoto (Yamamoto *et al.* 1988) has shown that the killer toxin of *W. mrakii* inhibits β (1-3)-glucan synthesis in sensitive yeast cells (although the mechanism by which this occurs is not yet known) and in terms of biological control Jijakli (Jijakli and Lepoivre 1998) reported that β-1,3-exo-glucanase activity was major mode of action for the antagonism between the yeast *P. anomala* strain K and *B. cinerea* on postharvest apples. Therefore, a method of fractionation using FPLC was employed to further characterise the secreted antifungal compounds from the yeast *P. anomala* NCYC 750.

The FPLC fractionation of the *P. anomala* NCYC 750 concentrated cell free supernatant (Section 5.6) again established the presence of proteins in the supernatant with three collections of absorption peaks observed. Enzyme assays performed on the FPLC fractionations (Section 5.71) of the concentrated cell free supernatant also showed exo-glucanase activity with several fractions all in the second pooled peak showing the greatest activity. However, when the three pooled FPLC fractions were tested for antimycotic activity using a microtitre assay (Section 5.72) the fraction showing the strongest effect on all three fungi tested was the pooled fraction three. Although not as pronounced as that exhibited by fraction three, fraction two did exhibit fungal growth inhibition but on only two of the fungi tested, namely *T. versicolor* and *P. placenta,* and was the least effective of all three fractions when assayed against *C. globosum.* Fraction three did appear to contain protein as a strong absorbance at 280 nm was observed during elution from the FPLC column and as this fraction was eluted at the end of the fractionation process it should contains the smallest proteins secreted by the yeast *P. anomala* NCYC 750.

In the microtitre assay of the fractionated *P. anomala* NCYC 750 concentrated cell free supernatant (Section 5.72) against *C. globosum* and *P. placenta* (Figures 5.11 and 5.13) anomalous results were obtained for the control preparation containing sterile water. Fungal growth in these two assays appeared to be significantly greater in the water control than in the malt extract control. This result could be due to the anomalous growth of fungi as discussed previously in this section, and therefore the use of more replicates may alter the results reported, or may be due to some stress caused by the sterile water resulting in the fungi exhibiting unusual or altered growth (when compared to the other treatments within the assay) which may be giving high absorbance readings.

The SDS-PAGE gel electrophoresis results reported in Section 5.81 (and Figure 5.14) may explain these results as it would appear that the fractionation of the cell free extract was not as pronounced as expected and some proteins of similar sizes were evident in the pooled fractions two and three. In fact a protein bands of 26 kDa was recorded in both fractions and a band of similar size (28 kDa) was evident in the unfractionated concentrated cell free supernatant. This single protein may be responsible for the antifungal activity, the exo-glucanse activity or both observed in the previous assays and could be the antifungal agent secreted by *P. anomala* NCYC 750. The lack of protein found in the pooled FPLC fraction one was probably due to low concentrations of protein collected as indicated by the low peak on the FPLC trace.

Chapter 6

Small scale simulated fence post assay of putative antifungal agents from killer yeasts

6.1 Introduction

In the previous chapters the potential for biocontrol of wood decay fungi using yeast was demonstrated. However all of the experiments performed and described in this study so far have been undertaken using artificial media under sterile laboratory conditions. Therefore, in order to assess the true potential of the yeast *P. anomala* NCYC 750 for use in the biocontrol of wood decay fungi, an experiment was performed in field conditions using wood as a fungal growth medium. Large scale wood decay field studies are usually undertaken over long time periods (6 months to many years) in order to allow significant and measurable decay to take place (Bruce *et al.* 1984; Bruce and King 1986a; Bruce and King 1986b; Bruce and Highley 1991; Ejechi 1997). As such small scale laboratory based experiments, simulating field conditions, are often employed in order to allow short term experimentation, but still provide useful data on potential biocontrol agents of wood decay (Highley and Ricard 1988; Pearce 1990; Schoeman *et al.* 1994; Ejechi and Obuekwe 1996; Canessa and Morrell 1997; Highley 1997)

In a standard field trial, treated and untreated wooden fence posts would be placed in a field plot or in large tubs containing soil. These fence posts would be monitored visually for fungal growth at regular intervals. After a period of at least four months the fence posts would be removed and the extent of wood decay would be assessed through

weight loss and visual observation. The length of time the fence posts are left in the field needs to be at least four months, and possibly much longer, in order that significant decay is observed in the untreated posts and therefore statistical differences can be measured between treated and untreated posts. A long time period is also useful as biological wood decay treatments need to be effective in the long term in order to minimise the number of reapplications and as such, be competitive with chemical treatments. A simulated fence post assay may be performed in large tubs containing sterile soil in an environmentally controlled laboratory environment (Brown and Bruce 1999; Brown *et al.* 1999). The use of these tubs would allow the introduction of a single fungal species into the soil in order to assess the putative biocontrol agents ability to inhibit this specific fungus.

In the small scale simulated fence post assay performed in this study the above conditions were replicated in 30ml universal bottles as described below. This allowed for a shorter length experiment as, even though only a small amount of wood decay would take place in this time period, a significant weight loss should be measurable due to the original size of the would blocks used.

6.2 **Small scale simulated fence post assay - Experimental approach**

The simulated fence post assay was performed in 30 ml universal bottles which were sterilised by autoclaving at 125°C and 15 psi for fifteen minutes. Soil was obtained from the SIWT fungal cellar and was sifted prior to use to remove any large particles. Sterile soil was obtained by autoclaving small volumes (approximately 200g) of the sifted soil at 121° and 15psi for 20 minutes, the soil was then left for 48 hours to allow any fungal spores to germinate and was then re-autoclaved at 121° and 15psi for 20 minutes.

The soil moisture content of sterile and non-sterile soil was adjusted to 95% of its water holding capacity using the method described in Chapter 2 (Section 2.9). Each universal was then filled approximately one third full with either sterile or non-sterile soil.

The wood used to simulate fence posts was beech (*Fagus sylvatica* L.) wood which was cut into strips measuring 10mm by 5mm in cross section, these strips were then cut into 30mm lengths to produce blocks of 5x10x30 mm in the tangential, radial and longitudinal directions respectively. Each block was numbered, weighed and sterilised by autoclaving for 20 minutes at 121°C and 15 psi. The wood decay fungus used was a seven day culture of the soft rot fungus *C. globosum* grown on malt extract agar (5%/2%) (Section 2.21). The simulated fence post assay was split into two sections using either sterile or non-sterile soil with seven replicates of each treatment and was performed as follows:

The preweighed sterile wood blocks were treated by submersion for 1 minute in one of the five treatments (a – e) listed below (concentration of FPLC fractions was performed by lyophilisation of a known volume of the fraction and resuspension in a 10 fold decreased volume):

a. 10 x concentrated FPLC fraction 3 in citrate buffer.
b. 10 x concentrated FPLC fraction 2 in citrate buffer.
c. 10x concentrated FPLC fraction containing no protein (ie a flat portion of the FPLC trace).
d. Citrate buffer.
e. Sterile distilled water.

The wood blocks were then aseptically placed onto the surface of the soil in the universal bottles. In the section of this simulated fence post assay using bottles containing sterile soil, a 4mm core of the fungus *C. globosum* was placed into the bottle next to the wood block. The bottles were then sealed and incubated in a fungal cellar at 21°C for 14 weeks (these conditions do not simulate environmental conditions but were used in order to stimulate fungal growth and therefore decay).

After this period the wooden blocks were removed from the bottles. Any soil and fungi clinging to the surface was brushed off and the blocks were weighed. The blocks were then dried overnight at 105°C and were re-weighed in order to calculate their dry weight. This dry weight was then subtracted from original weight recordings for each block, thus calculating the weight loss caused by fungal degradation on each block.

6.3 **Results of small scale simulated fence post assay**

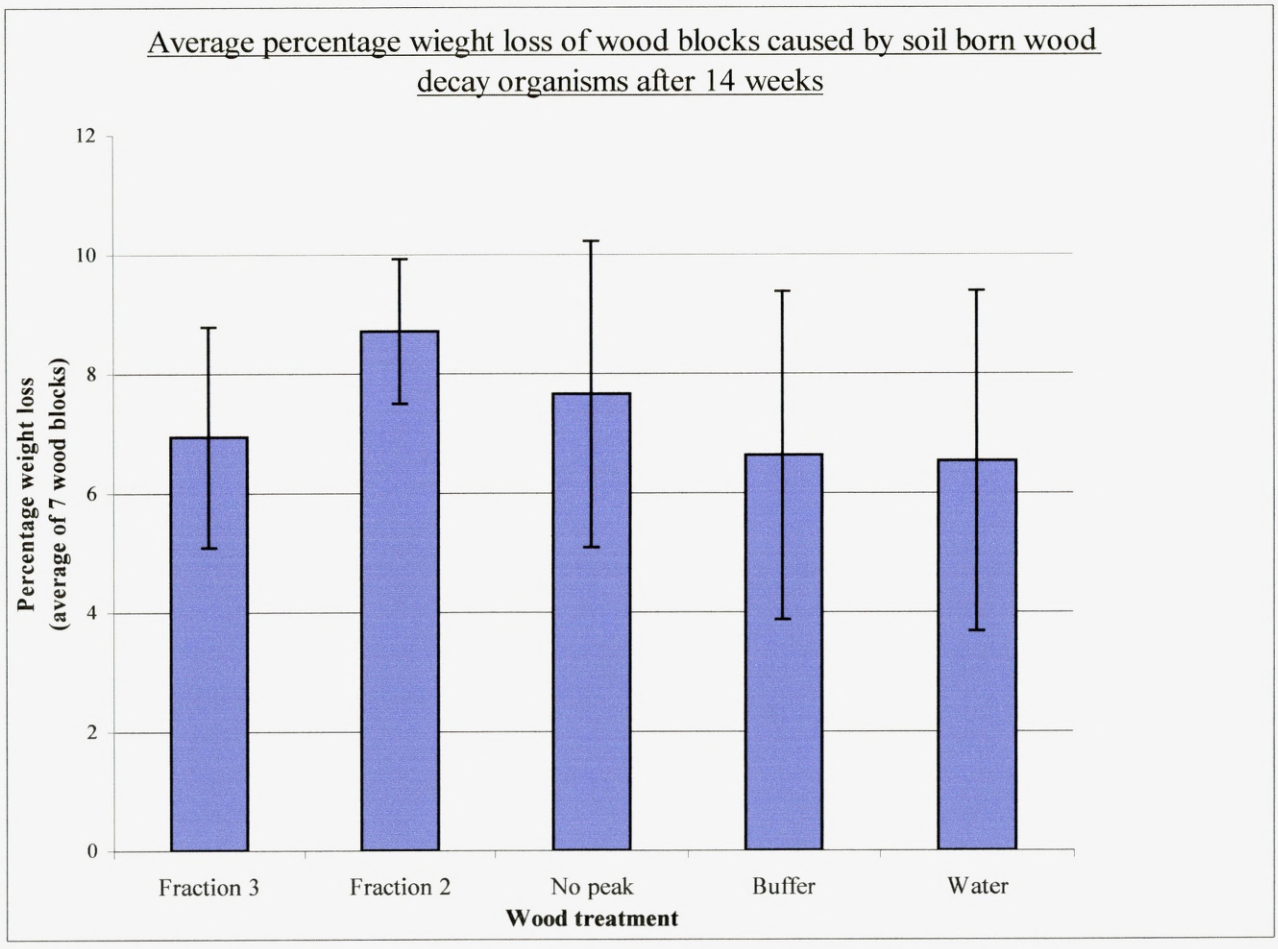
The average weight loss for each treatment in both the pure culture *Chaetomium* and non sterile soil conditions assayed are displayed in Figure 6.1 and Figure 6.2. The sample standard deviation was calculated for each data set and is displayed as error bars in the figures. An analysis of variance was performed on the data sets for each treatment (*Chaetomium* and non-sterile) and no significance was found, *Chaetomium* – $F(2.66) = 1.16$, $p = 0.35$, non sterile $F(2.67) = 1.08$, $p = 0.38$.

These results, from the small scale fence post assay, showed that the various treatments assayed had little statistically significant effect on the decay caused by natural soil born wood decay organism or the decay caused by the soft rot fungus *C. globosum*.

In sterile conditions the fungus *C. globosum* caused the most decay in the buffer control with an average weight loss of 8.3%. The decay in the wood blocks treated with fraction 3 and fraction 2 of the pooled FPLC sample was less than this at 7.5 and 7.9% weight loss respectively. The average decay in the wood blocks treated with the flat no-peak control fraction from the FPLC samples was recorded at a 6.3% weight loss. However, the minimum decay recorded was in the water control treatment with an average weight loss of 5.8%.

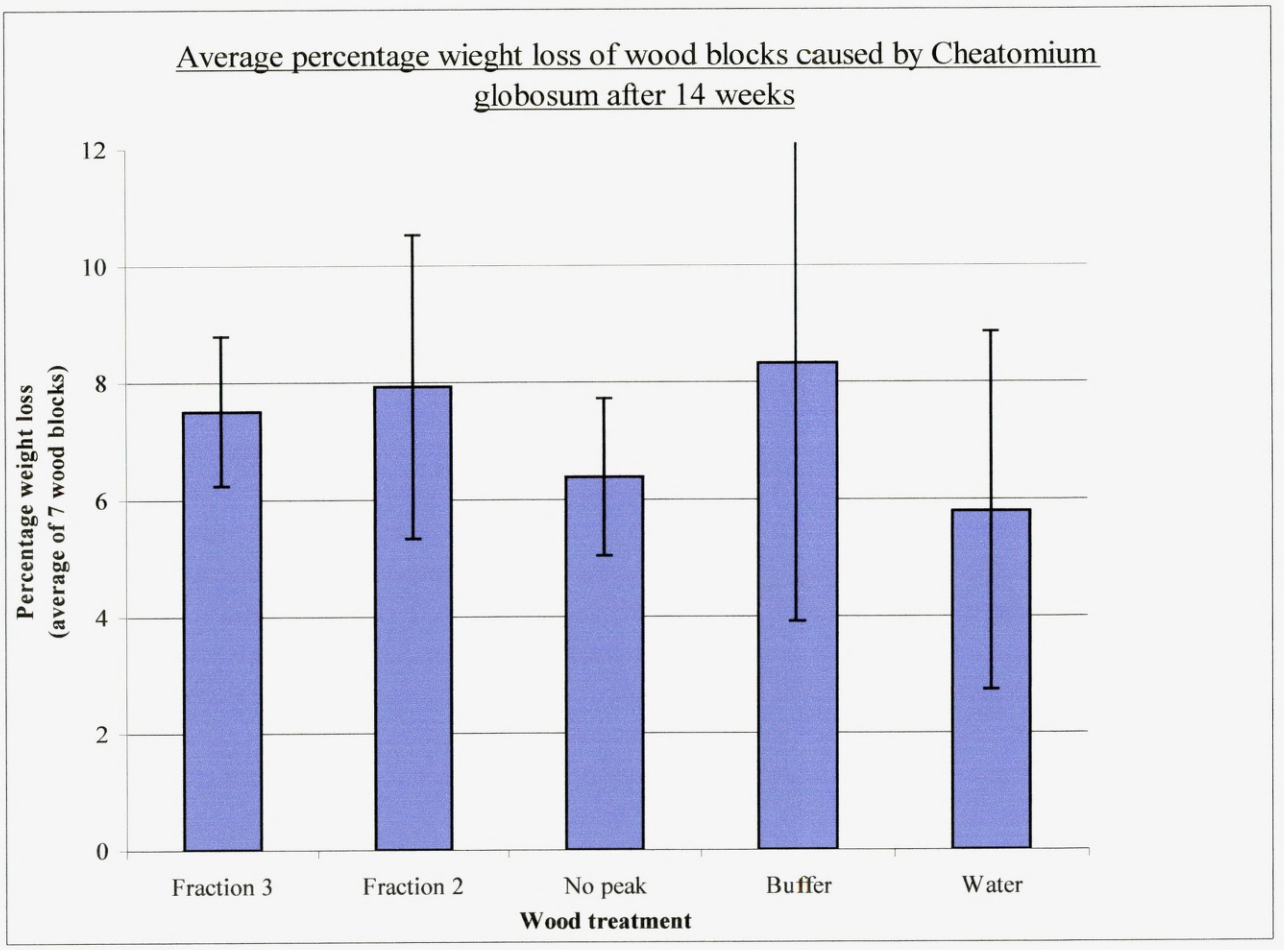
In the non sterile conditions the buffer and water controls with average percentage weight loss of 6.6% and 6.5% respectively had the least weight loss recorded, whilst fraction3 and the no-peak fraction recorded average weight losses of 6.9% and 7.7% respectively. The highest average weight loss of 8.7% was recorded by the fraction 2 treated blocks.

Figure 6.1 Results of small scale simulated fence post assay of secreted products from *P. anomala* NCYC 750 and their ability to prevent wood decay caused by soil organisms.



Error bars shows show standard deviation in replicate samples for each treatment

Figure 6.2 Results of small scale simulated fence post assay of secreted products from *P. anomala* NCYC 750 and their ability to prevent wood decay caused by the soft rot fungi *C. globosum.*



Error bars shows show standard deviation in replicate samples for each treatment

6.3. **Discussion**

The small scale fence post assay to show the possible effectiveness of the putative mycocin (antifungal agent) produced by the yeast *P. anomala* NCYC 750 was inconclusive in its findings. The simulated fence post assay tested two pooled FPLC fractions that had in laboratory assays inhibited fungal growth. One of the same fungi used in the laboratory was used in this fence post assay namely, *C. globosum* a soft rot fungus. The FPLC fractions did appear to slightly inhibit wood decay caused by this fungus when compared with a buffer control treatment but did not inhibit decay in comparison with a water control. A third fraction collected from the FPLC fractionation of the concentrated supernatant from *P. anomala* NCYC 750 but not containing protein was also tested. This fraction also appeared to slightly inhibit wood decay and to a greater extent than the putative toxin containing fractions.

The small scale simulated fence post assay also assayed the ability of the putative mycocin to inhibit wood decay caused by natural soil borne wood decay organisms in unsterilised soil. In this simulated fence post assay the least amount of weight loss observed and thus wood decay was in wood treated with the buffer and water control solutions. The wood treated with FPLC fraction 2 containing a putative mycocin actually recorded the highest decay of all. Wood treated with the other FPLC fraction containing a putative mycocin (fraction 3) did appear to be protected from wood decay to some extent with a nearly two percent less weight loss recorded than the fraction 2 treated wood but this was still marginally more decay than the controls. The wood treated with the no-peak FPLC fraction had less weight loss than fraction 2 treated wood but more than fraction 3 treated wood.

Due to the fact that most of the treated samples had more decay than the control samples, the high standard deviation and the lack of significance found in the analysis of variance, it would be hard to suggest that any biocontrol activity was recorded by this assay. This lack of antifungal activity when compared with the *in vitro* studies described in previous chapters was probably due to differences in conditions between the wood and *in vitro* (agar) conditions such as differences in pH and moisture content between agar and wood. These differences in physical conditions between the wood and agar may have caused the breaking down, and thus inactivation, of the putative antifungal agents, especially if this agent is enzymatic. The lack of biocontrol activity may also have been brought about through binding of the antifungal agent to substrates within the wood or through the leaching of the antifungal agent from the wood into the soil. However, in the non sterile trial the fraction 3 treated wood did appear to have less decay than the fraction 2 treated wood and this may indicate some antifungal protection had occurred during the trial. This difference in decay between the two treatments can also be seen, although to a much lesser extent, in the *C. globosum* trial. These factors and the results obtained with the no-peak fraction will be discussed in the following chapter.

Chapter 7

Discussion

7.1 Yeast / **Fungal Interactions**

The results described in the previous six chapters clearly demonstrate that killer yeast species can exhibit an effect on the growth of fungi and as such confirm the observations made by Walker *et. al.* (Walker *et al.* 1995). These effects, observed in the initial agar diffusion bioassay screening (chapter 2), were varied with some yeasts appearing to stimulate fungal growth, others showing little or no effect and some yeast appearing to be able to cause the inhibition of fungal growth. A broad spectrum of action was not observed in any of the yeast with no single yeast being antagonistic to all of the fungi assayed and conversely no single fungus being inhibited by all of the yeasts assayed. The majority of the antagonistic behaviour observed came from two yeast species namely the *Saccharomyces* and *Pichia* yeast species and all except one of these yeast, namely *S. cerevisiae* NCYC 1006 were known killer yeasts.

Two representative killer yeasts, one from each of these genera, namely *S. cerevisiae* K28 and *P. anomala* NCYC 750, were chosen from these species for further study.

7.2 Volatile **Effects**

Volatile chemicals have been shown to inhibit fungal growth in various studies (Dennis and Webster 1971a; Moleyar and Narashimham 1986; Yuen *et al.* 1995; Abou-Zeid *et al.* 1998) especially in the case of wood decay fungi (Bruce *et al.* 1984; Bruce *et*

al. 1996; Wheatley *et al.* 1997) and a recent study showed the potential of yeast volatiles to inhibit the growth of sapstain fungi (Payne *et al.* 2000). From the data reported in chapter 4 it would appear that volatile chemicals secreted by *P. anomala* NCYC 750 and *S. cerevisiae* K28 can be inhibitory to fungal growth. This may be especially so in the case of yeast from the *S.* species which may be fermenting on the malt extract broth and producing ethanol and higher alcohols. Ethanol is a substance known to inhibit fungal growth in low concentrations (0.5-1%) which could feasibly have been produced by the fermenting *S. cerevisiae* yeasts. Ethanol and acetaldehyde have been shown to inhibit the growth of postharvest disease causing organisms such as *Penicillium italicum* and *P. digitatum* (Yuen *et al.* 1995). In the case of the yeast from the *Pichia* species this effect may not be so easily explained because *Pichia* spp. are not as effective fermenters as *S.* yeasts. An antifungal effect was observed however in the volatile assays of *P. anomala* NCYC 750 versus wood decay fungi (Chapter 4) although this effect was not as pronounced as the inhibition caused by *S. cerevisiae* K28. Therefore some antifungal volatile chemicals must be produced by *P. anomala* NCYC 750. These chemicals could be secreted metabolites from normal anabolic processes such as acetaldehyde or ethyl acetate, as both of these compounds have been shown to inhibit fungal growth (Yuen *et al.* 1995; Fredlund *et al.* 2001), or could be unique antifungal chemicals, the production and secretion of which are stimulated during the natural ecological interactions between this yeast and other microorganisms. Further identification of secreted yeast volatile compounds and the screening of these compounds for antifungal activity must be undertaken for the elimination or further development of this concept as a biocontrol mechanism.

7.3 Mechanism of action of the antifungal agents secreted by yeast

The volatile effect, discussed above, although probably part of the mode of action would not explain the results observed in the later assays (chapter 5). Volatile effects could not explain the observations in the microtitre assays as: 1) any truly volatile compounds will have been lost to the air during the fermentation and subsequent media processing and 2) the process of lyophilisation performed prior to microtitre assays would have driven off any remaining volatile compounds including ethanol. Therefore, a secondary or entirely separate mechanism must be responsible for the fungal inhibition observed in the later assays.

From the data obtained in the initial screens some form of nutrient competition could explain the inhibition of fungal growth observed. Competition for carbon and nitrogen sources is known to inhibit spore germination in some fungi (Blakeman and Brodie 1977) and yeast have been shown to inhibit spore germination on apples (Jijakli *et al.* 1993b). The superior uptake of sugars by yeast has also been reported to inhibit the growth of the fungi *B. cinerea* by yeast (Filonow 1998) and signs of nutrient depravation have been observed in *B. cinerea when* grown with the yeast *C. saitoana* (El-Ghaouth *et al.* 1998). As a result of these observations nutrient competition has been suggested as a mode of action in the study of yeast as a biological control agent of postharvest fruit decay (Droby *et al.* 1989; Chand-Goyal and Spotts 1997). This mode of action may explain some of the results observed in the initial screens (chapter 3) of this thesis, but cannot explain the results obtained from the volatile, liquid and microtitre assays described in later chapters. Yeast cells did not come into contact with the fungal growth

media in the volatile assays and the latter assays involved the use of a yeast supernatant containing no cells therefore no nutrient competition could have taken place.

Therefore a third mechanism must be responsible for the antifungal results obtained in the microtitre and liquid assays. One possibility for this mechanism is that some metabolite secreted by yeast such as an antimycotic agent is acting as an inhibitor of fungal growth. This mechanism has been observed in the postharvest biocontrol of fungal decay on fruits with the bacterium *B. subtilus* which secretes an antimycotic compound inhibitory to the growth of a variety of fungi (Jiang 1997). Yeasts have been observed to secrete antibiotic agents as in the case of killer toxins but no studies of yeast fungal antagonisms in the field of biocontrol have found evidence for antimycotic activity in cell free extracts. However, this failure to discover antimycotic products in postharvest biocontrol of fruit caused by yeast could be because unrefined cell free extract have been used (Chand-Goyal and Spotts 1996) and the antimycotic agents secreted into a liquid growth media may be much more dilute than on a fruit surface and their production on fruit may be stimulated by the presence of fungi or by the fruit itself. Therefore the observations made in this thesis that a one hundred fold concentration of cell free extract of yeast supernatant may exhibit antifungal activity could be a mode of action for postharvest biocontrol of fruit decay. This antimycotic activity may not have been observed in previous studies due to insufficient concentration of supernatants from the biocontrol yeast.

In the biocontrol of wood decay, fungi from the genus *Trichoderma* have been shown to be effective biocontrol agents (in the laboratory) and these fungi have been shown to exhibit a variety of antagonistic behaviours including the secretion of

metabolites exhibiting antimycotic activity (Claydon *et al.* 1987; Bruce and Highley 1991; Highley 1997). Some of these antimycotic agents have been shown to be associated with chitinase and exo-glucanase (laminarinase) activity (Bruce *et al.* 1995). Chitinase activity has also been observed to be associated with the killer activity of yeast from the genera *Pichia* (McCracken *et al.* 1994) and Kluyveromyces (Butler *et al.* 1991b).

Therefore the third antifungal mode of action of *P. anomala* NCYC 750 and *S. cerevisiae* K28 may be due to the secretion of a proteinaceous antimycotic agent which exhibits chitinase or exo-glucanase activity. The enzyme assays described in chapter 5 showed no chitinase activity in the concentrated cell free supernatant of *P. anomala* NCYC 750 and *S. cerevisiae* K28, no exo-glucanase activity for *S. cerevisiae* K28 but did show exo-glucanase activity for *P. anomala* NCYC 750.

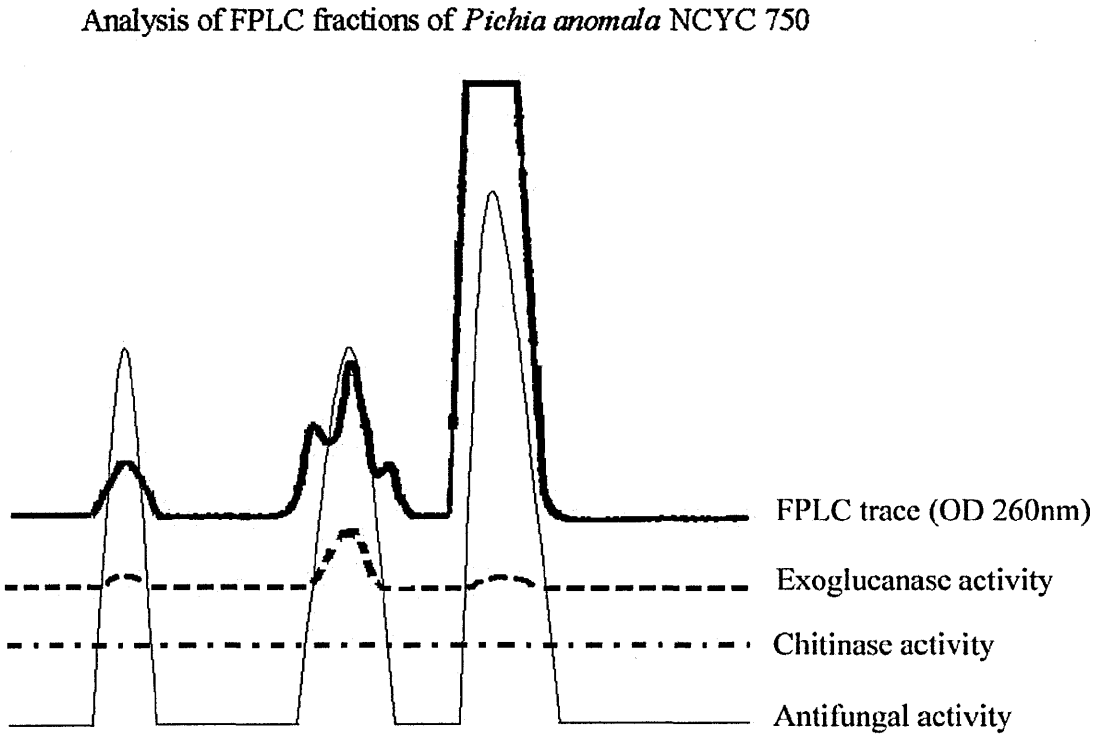
7.31 Mechanism of action of antifungal effects of *P. anomala* NCYC 750

Exo-β-1,3-glucanase activity has previously been reported in the yeasts *Cryptochoccus laurentii* (Castoria *et al.* 1997), *P. guilliermondii* (Wisniewski *et al.* 1991), *P. anomala* (Grevesse *et al.* 1998b) and *R. glutinis* (Castoria *et al.* 1997). All of these yeasts have been shown to be potential biocontrol agents of fungi (Roberts 1990, Wisniewski *et al.* 1991; Jijakli *et al.* 1993; Elad *et al.* 1994) inhibiting the growth of the post harvest decay fungi *B. cinerea, P. digitatum* and *Penicillium expansum* and the wheat storage mold *P. roqueforti* (Petersson *et al.* 1999). The mechanism by which this antagonism is mediated has been linked to the effects of exo-β-1,3-glucanase secreted by the yeast breaking down cell wall components (Wisniewski *et al.* 1991; Castoria *et al.* 1997). This mechanism has been further elucidated from studies of *B. cinerea exposed* to

the enzyme of *P. anomala* strain K which showed morphological changes in growing germ tubes such as swelling and leakage of cytoplasmic fluid (Grevesse *et al.* 1998b). Additional studies have found the same exo-β-1,3-glucanase secreted by *P. anomala* strain K to be present on apples after inoculation with *P. anomala* strain K for the biocontrol *B. cinerea* (Jijakli and Lepoivre 1998). Therefore, the exo-glucanase (laminarinase) found to be secreted by *P. anomala* NCYC 750 in this study may be the "antimycotic" mechanism inhibiting fungal growth.

Fractionation of the cell free supernatant of *P. anomala* NCYC 750 (Chapter 5) allowed for the further identification of the secreted antimycotic compound. The fractions were pooled into three protein containing fractions each of which exhibited some degree of inhibitory effect on *C. globosum, T. versicolor* and *P. placenta.* Fractions 2 and 3 exhibited the greatest degree of activity against *C. globosum* and *T. versicolor* whilst fraction 1 appeared to be the most effective inhibitor of growth of *P. placenta.* Exo-glucanase activity was observed in the three fractions with fraction 2 exhibiting much more activity than fractions 1 and 3. This relationship exhibited between antimycotic activity, FPLC fractionation and enzyme activity is represented in Figure 7.1.

Figure 7.1 Relationship between FPLC fractions, enzyme activity and antimycotic activity



Key – FPLC trace from FPLC fractionation (Section 5.62)
Exo-glucanase activity from exo-glucanase assay of FPLC fractions (Section 5.711)
Chitinase activity from enzyme assays of cell free ultrafiltered extracts (Section 5.431)
Antifungal activity from microtitre assays (Section 5.721)

In the latter part of the results of this thesis (Section 5.8) an attempt was made to identify the size of the exo-glucanase enzyme thought to be at least partly responsible for the antimycotic activity exhibited by *P. anomala* NCYC 750. SDS-PAGE gel

electrophoresis was used to examine the three pooled FPLC fractions and the concentrated cell free extract. Numerous proteins were found in the concentrated sample and the pooled fractions 2 and 3 and a protein with a molecular mass approximately 26 kDa appeared in all three. As all three of these samples exhibited some degree of exo-glucanase activity this protein could be the exo-glucanase antifungal enzyme. Further investigations of these proteins using non-denaturing gel electrophoresis and the excision of bands for sequence and enzymatic analysis may answer these questions. No proteins were found in fraction one and this fraction also exhibited some exo-glucanase activity. This could be due to the protein exhibiting this activity being too dilute in the sample to take the silver stain and therefore appear on the gel.

7.311 *P. anomala* NCYC 750 antifungal activity = Killer toxin?

The killer toxin of *P. anomala* NCYC 750 has yet to be identified or its mode of action elucidated. Killer toxins of other yeast however have been identified and are known to be proteins or glycoproteins with molecular weights ranging from 10 to 110 kDa and often consist of more than one subunit. Therefore it is suggested that the antimycotic activity reported above could plausibly be, or be a subunit of a, killer yeast toxin with a mass of at least 27 kDa.

If this 27 kDa protein is a subunit of a yeast killer toxin the exo-glucanase activity observed for the pooled FPLC fraction 1 (Section 5.711), which should contain the largest proteins secreted by the yeast *P. anomala* NCYC 750, may be a product of the larger complete killer toxin.

7.32 Mechanism of action of antifungal effects of *S. cerevisiae* K28

The mechanism by which *S. cerevisiae* K28 inhibits fungal growth was not identified in this study. However the mode of action was shown, at least in part to be associated with the secretion of volatile chemicals, probably involves some form of nutrient competition and is also in part mediated by a compound secreted during the growth of the yeast. This compound was not identified but could be a yeast killer toxin. The toxin of *S. cerevisiae* K28 is known to inhibit DNA synthesis through a complex process involving the binding of the toxin to a mannoprotein on the cell wall which facilitates the uptake of the toxin by endocytosis (Eisfeld *et al.* 2000), the toxin then passes through the cell via the yeast secretory process in reverse and inhibits DNA synthesis arresting cell growth at S phase of the cell cycle (Schmitt *et al.* 1996). This process could be occurring on the wood decay fungi used in this thesis and the killer toxin of *S. cerevisiae* may be the agent inhibiting fungal growth. Fungal cell walls do contain mannoprotein and as such the *S. cerevisiae toxin* could bind to fungi, however the 15 to 25% of the fungal cell wall is made up of chitin and is generally difficult to pass through. Endocytosis processes are not fully understood in fungi but probably do occur for nutrient uptake and are possibly more common in the growing hyphal tips of fungi. Therefore the yeast killer toxin of *S. cerevisiae* K28 could be taken up by fungi and may be the mechanism responsible for the fungal growth inhibition observed. This process may only be taking place in the growing hyphal tips of the wood decay fungi and therefore only preventing growth in these tips. This would explain the pattern of altered growth observed in the liquid assays using the non-fractionated cell free supernatant of *S. cerevisiae* K28. Further investigation using a labelled killer *S. cerevisiae* K28 toxin to

follow toxin uptake and assays for antifungal agents involving growth measurement of single fungal hyphae using optical microscopy and computer aided image analysis (Oh *et al.* 1993; Oh *et al.* 1995; Oh *et al.* 1996) may aid in the further elucidation of this effect.

7.4 **Mycoparasitism**

The inhibition of fungal growth caused by growing *S. cerevisiae* K28 and *P. anomala* NCYC 750 cells in the original screens (Chapter 3) and the liquid assay could be explained by the above factors and may be mediated through the attachment of yeast cells to the fungal hyphal walls. This phenomenon has been observed in yeast from the *Candida* genus which have been shown to bind to the hyphal walls of *B. cinerea* (Cook *et al.* 1997; El-Ghaouth *et al.* 1998) and cause severe cytological injury to the fungus and has been observed in a study of the biocontrol of *P. digitatum* by the yeasts *P. guilliermondii* and *R. glutinis* (Arras and Demontis 1996) and the biocontrol of *B. cinerea* by *P. guilliermondii* (Wisniewski *et al.* 1991). If this phenomenon was occurring, any antimycotic agents such as a killer toxin or exo-glucanase enzymes secreted by the yeast cells attached to fungal hyphae would be in much higher concentrations around these hyphae than in the surrounding media and therefore, any antifungal activity would be more effective. This could be easily investigated through the use of electron or optical microscopic analysis of fungi grown with yeast and the identification of fungal hyphal colonisation by yeast and any subsequent hyphal damage.

7.5 Potential of killer yeast as a biocontrol agent of wood decay fungi

As discussed in Section 1.14 the main difficulties with the development of biocontrol for wood decay is that any biological agent must be capable of complete colonisation of the wood, long term survival and be capable of maintaining antifungal efficacy in wood. A lack of complete colonisation and long term survival are probably the main factors responsible for the lack of successful transfer of proven laboratory biocontrol agents into the field. Due to the limited nutrient availability and low moisture content in wood it is doubtful that yeast cells could survive in effective biocontrol concentrations for long periods in wood. However the use of supplemental nutrients and/or low doses of conventional antifungal agents, as successfully used in the biological control of postharvest disease organisms (Chand-Goyal and Spotts 1996; El-Neshawy and Wilson 1996), may aid in the colonisation of the wood and long term efficacy of the yeasts.

A second problem associated with the use of biocontrol organisms in the prevention of wood decay is the timing of the application of the biocontrol agent, as in order to prevent fungal growth an organism may need to well established within the wood. Timber is often kiln dried prior to construction use or storage, this process sterilises the wood to some extent, therefore, leaving the wood open to primary colonisation by decay organisms if it subsequently becomes re-wetted. As such some mechanism to prevent this colonisation is needed. Inoculation of a biocontrol organism such as yeast after kiln drying however, would be extremely difficult due to the low moisture content of the wood preventing a thorough colonisation of the wood, especially the interior. Therefore inoculation and colonisation of the wood prior to drying would be

advantageous. In studies of yeast as biocontrol agents of postharvest disease of fruits yeast have been shown to be capable of surviving exposure to 60°C in drying tunnels (Chand-Goyal and Spotts 1997). This temperature is similar to that used in the kiln drying of wood although the length of time at this temperature is significantly longer and killing effect of heat is a temperature/time relationship. Consequently, yeast whole cell treatments may show some potential for use as biocontrol agents of wood decay organisms if inoculated prior to kiln drying although their ability to survive kiln drying would need to be established.

Some of the above difficulties involving the use of whole cells could be overcome if an antifungal metabolite could be isolated, purified and concentrated from the putative biocontrol agent. This purified "mycocin" could be used in a similar way to the conventional chemical treatments of wood decay. The identification of a putative antifungal metabolite secreted by yeast illustrated in this study may prove to be such a mycocin. However, it is doubtful that the mycocin could survive kiln drying as the majority of enzymes and killer toxins would be inactivated after prolonged exposure to temperatures of 60°C. As such any yeast mycocin would need to be applied post kiln dying using some form of pressure injection in order to ensure full coverage and thus protection of the treated wood. A simultaneous inoculation of a low dose of a conventional biocontrol agent or whole cell treatment may also increase efficacy.

The simulated fence post assay in this study (chapter 6) showed very little biocontrol activity. This may have been due to a number of reasons such as conditions within the wood inactivating the biocontrol agent, the putative mycocin may not have been at a high enough concentration for effective biocontrol to be seen or the simple

dipping of the wood blocks into the putative mycocin may only have provided surface protection and not prevented the internal decay of the blocks. In order to combat these effects the mycocin must be concentrated further prior to application onto the wood. This could be achieved through further lyophilisation or some form of selective filtration followed by lyophilisation. It may also be possible to stimulate mycocin production especially exo-glucanase by culturing yeast in the presence of fungal cell walls, as this has been shown to stimulate exo-glucanase production (Bruce *et al.* 1995; Grevesse *et al.* 1998a), and therefore produce a higher initial mycocin concentration.

7.6 **Conclusions**

This study has demonstrated that yeast may possess the potential to be used as biocontrol agents of wood decay. All of the yeasts assayed exhibited some form of antagonism to at least one wood decay fungus and yeasts from the *Saccharomyces* and *Pichia* genera exhibited strong antifungal effects. The mode of action by which this antagonism is mediated was studied further in two yeast species representative of these genera and a number of possible mechanisms were identified. The possible antifungal mechanisms identified include;

- Volatile chemical secretions by the two yeast species such as alcohols, acetaldehyde or ethyl acetate
- Nutrient competition, the yeasts may be able to utilize nutrients especially carbon or nitrogen more rapidly than the fungi and therefore deprive the fungi
- Secretion of an antimycotic agent, a 27 kDa exo-glucanase enzyme secreted by the yeast *P. anomala* NCYC 750 is probably this antimycotic agent.

- The reported antimycotic effect caused by an exo-glucanase enzyme with a mass of 27 kDa may be associated with a yeast killer toxin.

These mechanisms as well as putative mechanisms not specifically identified in this study are listed in Table 7.1 and are ranked in their theoretical importance to antifungal activity based on information obtained within the literature cited in this study and the findings of this study.

Table 7.1 Modes of action of yeast antimycotic activity and relative importance.

Mode of action	*Importance in antifungal activity*
Nutrient competition	*****
Media acidification	*
Volatile chemicals	****
Killer toxin (with chitinase activity)	***
Antimycotic enzymes	**
Predation/hyphal attachment	***

Key- Importance * = least - ***** = most

This potential of killer yeast to act as biocontrol agents of wood decay fungi was, however, only demonstrated in laboratory conditions and no *in vivo* activity was detected. As such the modes of action described above and their importance to antifungal activity may be completely different in a wood based system. Therefore, in order to elucidate this potential further, killer yeast or yeast must be studied further *in vivo*. These further

studies should focus on the treatment methods used to apply the biocontrol agent (yeast or secreted antifungal product) to the wood to be protected and must identify the long term stability and biocontrol activity of these treatments.

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